Attorney's Docket No.: 08191-018001

APPLICATION

FOR

UNITED STATES LETTERS PATENT

TITLE: DELIVERY SYSTEMS FOR BIOACTIVE AGENTS

APPLICANT: SHIKHA P. BARMAN, UNA MCKEEVER AND MARY LYNNE HEDLEY

CERTIFICATE OF MAILING BY EXPRESS MAIL

Express Mail Label No. EF353815399US

I hereby certify under 37 CFR §1.10 that this correspondence is being deposited with the United States Postal Service as Express Mail Post Office to Addressee with sufficient postage on the date indicated below and is addressed to the Commissioner for Patents, Washington, D.C. 20231

June 1, 2001
Date of Deposit

Signature

Francisco Robles
Typed or Printed Name of Person Signing Certificate

DELIVERY SYSTEMS FOR BIOACTIVE AGENTS

Cross-Reference To Related Application

The application claims priority from U.S. Provisional Application Serial Number 60/208,830 filed on June 2, 2000.

Background of the Invention

This invention relates to methods of delivering nucleic acids into cells.

Gene therapy is a highly promising technique for treatment of hereditary diseases, e.g., cystic fibrosis. Gene therapy can also be used when expression of gene products from genes that are not naturally found in the host cells is desired, for example, from genes encoding cytotoxic proteins targeted for expression in cancer cells. Gene therapy can fall into several categories. It is sometimes desirable to replace a defective gene for the entire lifespan of a mammal, as in the case of an inherited disease such as cystic fibrosis, phenylketonuria, or severe combined immunodeficiency disease (SCID). In other cases, one may wish to treat a mammal with a gene that will express a therapeutic polypeptide for a limited amount of time, e.g., during an infection. Nucleic acids in the form of antisense oligonucleotides or ribozymes are also used therapeutically. Moreover, polypeptides encoded by nucleic acids can be effective stimulators of the immune response in mammals.

Various techniques have been used for introducing genes into cells, including infection with viral vectors, biolistic transfer, injection of "naked" DNA (US Patent No. 5,580,859), and delivery via liposomes or polymeric particles.

Summary of the Invention

The invention is based on the discovery that a delivery matrix containing an anionic or zwitterionic compound and a bioactive agent are highly effective vehicles for the delivery of bioactive agents into cells.

In general, the invention features a composition containing a delivery matrix, an anionic compound, and a bioactive agent, e.g. a peptide, protein, or nucleic acid, e.g., a nucleic acid described herein.

In a preferred embodiment, the delivery matrix includes a polymer, an oligomer, or a small molecule. Preferably, the delivery matrix is a microparticle, a hydrogel, an emulsion, a solution, a solid, a dispersion, or a complex.

In a preferred embodiment, the anionic compound has a pKa of less than about 4.5, preferably less than about 2.5, more preferably less than about 2.0, and most preferably about 1.8. Preferably, the anionic compound includes a phosphate, phosphonate, sulfate, or sulfonate.

Examples of anionic compounds useful in the invention include polyethylene glycol diacyl ethanolamine, taurocholic acid, taurodeoxycholic acid, chrondoitin sulfate, alkyl phosphocholines, alkyl-glycero-phosphocholines, phosphatidylserine, phosphotidylcholine, phosphotidylinositol, cardiolipin, lysophosphatide, sphingomyelin, phosphatidylglycerols, phosphatidic acid, diphytanoyl derivatives, glycocholic acid, cholic acid, and N-lauroyl sarcosine.

In a preferred embodiment, the anionic compound is a component of the delivery matrix. Examples of delivery matrices of the invention that contain an anionic compound as a component include a synthetically modified phosphonate derivatized macrocycle, a synthetically modified sulfonate derivatized macrocycle, a synthetically modified phosphonate derivatized cyclodextrin, and a synthetically modified sulfonate derivatized cyclodextrin.

In a preferred embodiment, the delivery matrix includes a synthetically modified phosphonate polymeric derivative. Preferably the synthetically modified phosphonate polymeric derivative is a rotaxane or a polymacrocycle.

In another preferred embodiment, the delivery matrix includes a synthetically modified sulfonate polymeric derivative. Preferably the synthetically modified sulfonate polymeric derivative is a rotaxane or a polymacrocycle.

In another aspect, the invention includes a composition containing a delivery matrix, a zwitterionic compound, and a bioactive agent, e.g. a peptide, protein, or nucleic acid, e.g., a nucleic acid described herein. In a preferred embodiment, the zwitterionic compound includes a phosphate, phosphonate, sulfate, or sulfonate.

In a preferred embodiment, the delivery matrix includes a polymer, an oligomer, or a small molecule. Preferably, the delivery matrix is a microparticle, a hydrogel, an emulsion, a solution, a solid, a dispersion, or a complex.

In a preferred embodiment the zwitterionic compound includes CHAPSO (3-3-(cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate), CHAPS ((3-3-(cholamidopropyl)dimethylammonio]- 1-propanesulfonate, poly(AMPS) (poly(2-acrylamido-2-methyl-1-propanesulfonic acid), or phosphatidylethanolamine.

In a preferred embodiment, the zwitterionic compound is a component of the delivery matrix. Examples of delivery matrices of the invention that contain a zwitterionic compound as a component include a synthetically modified phosphonate derivatized macrocycle, a synthetically modified sulfonate derivatized macrocycle, a synthetically modified phosphonate derivatized cyclodextrin, and a synthetically modified sulfonate derivatized cyclodextrin.

In a preferred embodiment, the delivery matrix includes a synthetically modified phosphonate polymeric derivative. Preferably the synthetically modified phosphonate polymeric derivative is a rotaxane or a polymacrocycle.

In another preferred embodiment, the delivery matrix includes a synthetically modified sulfonate polymeric derivative. Preferably the synthetically modified sulfonate polymeric derivative is a rotaxane or a polymacrocycle.

In one aspect, the invention includes a microparticle, e.g. a microcapsule or a microsphere, containing a polymeric matrix, an anionic lipid, and a nucleic acid molecule, e.g. a nucleic acid molecule described herein. Preferably, the microparticle is not encapsulated in a liposome and the microparticle does not comprise a cell or a virus. Preferably the microparticle is less than about 100 microns in diameter, more preferably less than about 60 microns in diameter, most preferably about 50 microns in diameter. In other embodiments, the microparticle is less than about 20 microns in diameter, or less than about 11 microns in diameter. Preferably the lipid has a pKa of less than about 4.5, preferably less than about 2.5, more preferably less than about 2.0, and most preferably about 1.8.

In a preferred embodiment the lipid is a lipid sulfonate, lipid sulfate, lipid phosphonate, or lipid phosphate. Examples of lipids of the invention include polyethylene glycol diacyl ethanolamine, taurocholic acid, glycocholic acid, cholic acid, N-lauroyl sarcosine, and phosphatidylinositol. Preferably the lipid is polyethylene glycol diacyl ethanolamine or taurocholic acid.

In another aspect, the invention includes a microparticle, e.g. a microcapsule or a microsphere, containing a polymeric matrix, a zwitterionic lipid, and a nucleic acid molecule, e.g. a nucleic acid molecule described herein. Preferably, the microparticle is not encapsulated in a liposome and the microparticle does not comprise a cell. Preferably the microparticle is less than about 100 microns in diameter, more preferably less than 20 microns in diameter, and most preferably less than 11 microns in diameter.

Examples of zwitterionic lipids of the invention include CHAPSO (3-3-(cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate), CHAPS ((3-3-

(cholamidopropyl)dimethylammonio]- 1-propanesulfonate, and phosphatidylethanolamine.

Microparticles of the invention are highly effective vehicles for the delivery of polynucleotides into phagocytic cells. "Microparticles" include both microspheres and microcapsules, e.g. hollow spheres.

In one aspect, the invention features a microparticle less than about 100 microns in diameter (e.g., about 100 microns, between 60 and 100 microns, less than about 60 microns, less than about 50 microns, less than about 40 microns, less than about 30 microns, less than about 20 microns, less than about 11 microns, less than about 5 microns, or less than about 1 micron), including a polymeric matrix and nucleic acid. The polymeric matrix preferably includes one or more synthetic polymers having solubility in water of less than about 1 mg/l; in the present context, synthetic is defined as non-naturally occurring. The nucleic acid is either RNA, at least 50% (and preferably at least 70% or even 80%) of which is in the form of closed circles, or circular DNA plasmid molecules, at least 25% (and preferably at least 35%, 40%, 50%, 60%, 70%, or even 80%) of which are supercoiled. The plasmid can be linear or circular. When circular and double-stranded, it can be nicked, i.e., in an open circle, or super-coiled. The nucleic acid, either single-stranded or double-stranded, can also be in a linear form.

The polymeric matrix is made from one or more synthetic polymers having a solubility in water of less than about 1 mg/l. At least 50% (and preferably at least 70% or even 80%) of the nucleic acid molecules are in the form of supercoiled DNA.

The polymeric matrix can be biodegradable. "Biodegradable" is used here to mean that the polymers degrade over time into compounds that are known to be cleared from the host cells by normal metabolic pathways. Generally, a biodegradable polymer will be substantially metabolized within about 1 month after injection into a patient, and certainly within about 2 years. In certain cases, the polymeric matrix can be made of a single synthetic, biodegradable copolymer, e.g., poly-lactic-*co*-glycolic acid (PLGA).

The ratio of lactic acid to glycolic acid in the copolymer can be within the range of about 1:2 to about 4:1 by weight, preferably within the range of about 1:1 to about 2:1 by weight, and most preferably about 65:35 by weight. In some cases, the polymeric matrix also includes a targeting molecule such as a ligand, receptor, or antibody, to increase the specificity of the microparticle for a given cell type or tissue type.

For certain applications, the microparticle has a diameter of less than about 11 microns. The microparticle can be suspended in an aqueous solution (e.g., for delivery by injection or orally) or can be in the form of a dry solid (e.g., for storage or for delivery via inhalation, implantation, or oral delivery). The nucleic acid can be an expression control sequence operatively linked to a coding sequence. Expression control sequences include, for example, any nucleic acid sequences known to regulate transcription or translation, such as promoters, enhancers, or silencers. In preferred examples, at least 60% or 70% of the DNA is supercoiled. More preferably, at least 80% is supercoiled.

In another embodiment, the invention features a microparticle less than about 100 microns in diameter (e.g., about 100 microns, between 60 and 100 microns, less than about 60 microns, less than about 50 microns, less than about 40 microns, less than about 30 microns, less than about 20 microns, less than about 11 microns, less than about 5 microns, or less than about 1 micron), including a polymeric matrix and a nucleic acid molecule (preferably in closed, circular form), wherein the nucleic acid molecule includes an expression control sequence operatively linked to a coding sequence. The expression product encoded by the coding sequence can be a polypeptide at least 7 amino acids in length, having a sequence essentially identical to the sequence of either a fragment of a naturally-occurring mammalian protein or a fragment of a naturally-occurring protein from an agent that infects or otherwise harms a mammal; or a peptide having a length and sequence that permit it to bind to an MHC class I or II molecule. Examples are set forth in WO 94/04171, hereby incorporated by reference.

"Essentially identical" in the context of a DNA or polypeptide sequence is defined here to mean differing no more than 25% from the naturally occurring sequence, when the closest possible alignment is made with the reference sequence and where the differences do not adversely affect the desired function of the DNA or polypeptide in the methods of the invention. The phrase "fragment of a protein" is used to denote anything less than the whole protein.

To determine the percent identity of two amino acid sequences or of two nucleic acids, the sequences are aligned for optimal comparison purposes (e.g., gaps can be introduced in the sequence of a first amino acid or nucleic acid sequence for optimal alignment with a second amino or nucleic acid sequence). The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences (i.e., % identity = # of identical positions/total # of positions (e.g., overlapping positions) x 100). Preferably, the two sequences are the same length.

The determination of percent homology between two sequences can be accomplished using the algorithm of Karlin and Altschul (1990) *Proc. Natl. Acad. Sci. USA* 87:2264-2268, modified as in Karlin and Altschul (1993) *Proc. Natl. Acad Sci. USA* 90:5873-5877. Such an algorithm is incorporated into the NBLAST and XBLAST programs of Altschul et al. (1990) *J. Mol. Biol.* 215:403-410. BLAST nucleotide searches can be performed with the NBLAST program, score = 100, wordlength = 12, to obtain nucleotide sequences homologous to a nucleic acid molecule of the invention. BLAST protein searches can be performed with the XBLAST program, score = 50, wordlength = 3, to obtain amino acid sequences homologous to a protein molecule of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can

be utilized as described in Altschul et al. (1997) *Nucleic Acids Res.* 25:3389-3402. Alternatively, PSI-Blast can be used to perform an iterated search that detects distant relationships between molecules. *Id.* When utilizing BLAST, Gapped BLAST, and PSI-Blast programs, the default parameters of the respective programs (e.g., XBLAST and NBLAST) should be used. *See* http://www.ncbi.nlm.nih.gov.

In calculating percent identity, only exact matches are counted.

The peptide or polypeptide can be linked to a trafficking sequence. The term "trafficking sequence" refers to an amino acid sequence that causes a polypeptide to which it is fused to be transported to a specific compartment of the cell, e.g., the nucleus, endoplasmic reticulum, the golgi apparatus, an intracellular vesicle, a lysosome, or an endosome. The term "trafficking sequence" is used interchangeably with "trafficking signal" and "targeting signal."

In the embodiment where the expression product includes a peptide having a length and sequence that permit it to bind an MHC class I or II molecule, the expression product is typically immunogenic. The expression product can have an amino acid sequence that differs from the sequence of a naturally occurring protein recognized by a T cell in the identity of not more than 25% of its amino acid residues, provided that it can still be recognized by the same T cell and can alter the cytokine profile of the T cell (i.e., an "altered peptide ligand"). The differences between the expression product and the naturally occurring protein can, for example, be engineered to increase cross-reactivity to pathogenic viral strains or HLA-allotype binding.

Examples of expression products include amino acid sequences at least 50% identical to the sequence of a fragment of myelin basic protein (MBP), proteolipid protein (PLP), invariant chain, GAD65, islet cell antigen, desmoglein, α-crystallin, or β-crystallin, where the fragment can bind the MHC class II molecule. Table 1 lists many of such expression products that are thought to be involved in autoimmune disease. Fragments of these proteins can be essentially identical to any one of SEQ ID NOS: 1-46

such as MBP residues 80-102 (SEQ ID NO: 1), PLP residues 170-191 (SEQ ID NO: 2), or invariant chain residues 80-124 (SEQ ID NO: 3). Other fragments are listed in Table 2.

Alternatively, the expression product can include an amino acid sequence essentially identical to the sequence of an antigenic portion of any of the tumor antigens listed in Table 3 such as those encoded by the human papilloma virus E1, E2, E6 and E7 genes, Her2/neu gene, the prostate specific antigen gene, the melanoma antigen recognized by T cells (MART) gene, or the melanoma antigen gene (MAGE). Again, the expression product can be engineered to increase cross-reactivity.

In still other cases, the expression product includes an amino acid sequence essentially identical to the sequence of an antigenic fragment of a protein naturally expressed by a virus, e.g., a virus that chronically infects cells, such as human papilloma virus (HPV), human immunodeficiency virus (HIV), herpes simplex virus (HSV), hepatitis B virus (HBV), or hepatitis C virus (HCV); a bacterium, such as mycobacteria; a fungi such as *Candida, Aspergillus, Cryptococcus,* or *Histoplasmosis* species, or other eukaryotes, such as a *Plasmodium* species. A representative list of such class I-binding fragments as well as fragments of tumor antigens is included in Table 4.

TABLE 1: Autoantigens

Disease	Associated Antigen	Notes
Coeliac disease	α-Gliadin	a
Goodpasture's syndrome	Basement membrane collagen	a
Graves' disease	Thyroid Stimulating Hormone (TSH) receptor	a
Hashimoto's disease	Thyroglobulin	a
Isaac's syndrome	voltage-gated potassium channels	b
Insulin-dependent diabetes	Glutamic acid decarboxylase (GAD)	a
	Insulin receptor	a
	Insulin associated antigen (IA-w)	a
	Hsp	b
Lambert-Eaton myasthenic syndrome (LEMS)	Synaptogamin in voltage-gated calcium channels	b

Multiple sclerosis	Myelin basic protein (MBP)	a
	Proteolipid protein (PLP)	a
	Myelin oligodendrocyte-associated protein (MOG)	a
	αB-crystallin	a
Myasthenia Gravis	Acetyl choline receptor	a
Paraneoplastic encephalitis	RNA-binding protein HuD	b
Pemphigus vulgaris	"PeV antigen complex"	a
	Desmoglein (DG)	c
Primary Biliary cirrhosis	Dihydrolipoamide acetyltransferase	b
	Pyruvate dehydrogenase complex 2 (PDC-E2)	d
Progressive systemic sclerosis	DNA topoisomerase	a
	RNA polymerase	a
Rheumatoid arthritis	Immunoglobulin Fc	a
	Collagen	
Scleroderma	Topoisomerase I	b
Stiff-man syndrome	Glutamic acid decarboxylase (GAD)	a
Systemic lupus erythematosus	ds-DNA	a
Uveitis	Interphotoreceptor retinoid-binding protein	b
	S antigen (rod out segment)	b

References:

a) HLA and Autoimmune Disease, R Heard, pg 123-151 in HLA & Disease, Academic Press, New York, 1994, (R. Lechler, ed.)
b) Cell 80, 7-10 (1995)
c) Cell 67, 869-877 (1991)
d) JEM 181, 1835-1845 (1995)

TABLE 2: Class II Associated Peptides

Peptide	SEQ ID NO:	Source Protein
GRTQDENPVVHFFKNIVTPRTPP	1	MBP 80-102
AVYVYIYFNTWTTCQFIAFPFK	2	PLP 170-191
FKMRMATPLLMQA	3	Invariant chain 88-100
TVGLQLIQLINVDEVNQIV TTNVRLKQQWVDYNLKW	4	Achr α 32-67
QIVTTNVRLKQQWVDYNLKW	5	Achr α 48-67

QWVDYNL	6	Achr α 59-65
GGVKKIHIPSEKIWRPDL	7	Achr α 73-90
AIVKFTKVLLQY	8	Achr α 101-112
WTPPAIFKSYCEIIVTHFPF	9	Achr α 118-137
MKLGTWTYDGSVV	10	Achr α 144-156
MKLGIWTYDGSVV	11	Achr α 144-157 analog(I-148)
WTYDGSVVA	12	Achr α 149-157
SCCPDTPYLDITYHFVM	13	Achr α 191-207
DTPYLDITYHFVMQRLPL	14	Achr α 195-212
FIVNVIIPCLLFSFLTGLVFY	15	Achr α 214-234
LLVIVELIPSTSS	16	Achr α 257-269
STHVMPNWVRKVFIDTIPN	17	Achr α 304-322
NWVRKVFIDTIPNIMFFS	18	Achr α 310-327
IPNIMFFSTMKRPSREKQ	19	Achr α 320-337
AAAEWKYVAMVMDHIL	20	Achr α 395-410
IIGTLAVFAGRLIELNQQG	21	Achr α 419-437
GQTIEWIFIDPEAFTENGEW	22	Achr γ 165-184
MAHYNRVPALPFPGDPRPYL	23	Achr γ 476-495
LNSKIAFKIVSQEPA	24	desmoglein 3 190-204
TPMFLLSRNTGEVRT	25	desmoglein 3 206-220
PLGFFPDHQLDPAFGA	26	HBS preS1 10-25
LGFFPDHQLDPAFGANS	27	HBS preS1 11-27
FFLLTRILTI	28	HBS Ag 19-28
RILTIPQSLD	29	HBS Ag 24-33
TPTLVEVSRNLGK	30	HSA 444-456
AKTIAYDEEARR	31	hsp 65 2-13
VVTVRAERPG	32	hsp 18 61-70
SQRHGSKYLATASTMDHARHG	33	MBP 7-27
RDTGILDSIGRFFGGDRGAP	34	MBP 33-52
QKSHGRTQDENPVVHFFKNI	35	MBP 74-93
DENPVVHFFKNIVT	36	MBP 84-97
ENPVVHFFKNIVTPR	37	MBP 85-99
HFFKNIVTPRTPP	38	MBP 90-102
KGFKGVDAQGTLSK	39	MBP 139-152
VDAQGTLSKIFKLGGRDSRS	40	MBP 144-163
LMQYIDANSKFIGITELKK	41	Tetanus Toxoid 828-846
QYIKANSKFIGIT	42	Tetanus Toxoid 830-842
FNNFTVSFWLRVPK	43	Tetanus Toxoid 947-960
SFWLRVPKVSASHLE	44	Tetanus Toxoid 953-967
KFIIKRYTPNNEIDSF	45	Tetanus Toxoid 1174-1189
GQIGNDPNRDIL	46	Tetanus Toxoid 1273-1284

TABLE 3: Tumor Antigens

Cancer	Associated Antigen
Melanoma	BAGE 2-10
Breast/Ovarian	c-ERB2 (Her2/neu)
Burkitt's lymphoma/Hodgkin's lymphoma	EBNA-1
Burkitt's lymphoma/Hodgkin's lymphoma	EBNA-2
Burkitt's lymphoma/Hodgkin's lymphoma	EBNA-3
Burkitt's lymphoma/Hodgkin's lymphoma	EBNA-3A
Burkitt's lymphoma/Hodgkin's lymphoma	EBNA-3C
Burkitt's lymphoma/Hodgkin's lymphoma	EBNA-4
Burkitt's lymphoma/Hodgkin's lymphoma	EBNA-6
Burkitt's lymphoma/Hodgkin's lymphoma	EBV
Burkitt's lymphoma/Hodgkin's lymphoma	EBV LMP2A
Melanoma	GAGE-1
Melanoma	gp75
Cervical	HPV 16 E6
Cervical	HPV 16 E7
Cervical	HPV 18 E6
Cervical	HPV 18 E7
Melanoma	MAG
Melanoma	MAGE-1
Melanoma	MAGE-2
Melanoma	MAGE-3
Melanoma	MAGE-4b
Melanoma	MAGE-5
Melanoma	MAGE-6
Melanoma	MART-1/Melan-A
Pancreatic/Breast/Ovarian	MUC-1
Melanoma	MUM-1-B
Breast/Colorectal/Burkitt's lymphoma	p53
Melanoma	Pmel 17(gp100)
Prostate	PSA Prostate Specific Antigen
Melanoma	Tyrosinase
	CEA Carcinoembryonic Antigen
	LRP Lung Resistance Protein
	Bcl-2
	Ki-67

TABLE 4: Class I associated tumor and pathogen peptides

Peptide	SEQ ID NO:	Source Protein
AARAVFLAL	47	BAGE 2-10
YRPRPRRY	48	GAGE-1 9-16
EADPTGHSY	49	MAGE-1 161-169
SAYGEPRKL	50	MAGE-1 230-238
EVDPIGHLY	51	MAGE-3 161-169
FLWGPRALV	52	MAGE-3 271-279
GIGILTV	53	MART-1 29-35
ILTVILGV	54	MART-1 32-39
STAPPAHGV	55	MUC-1 9-17
EEKLIVVLF	56	MUM-1 261-269
MLLAVLYCL	57	TYROSINASE 1-9
SEIWRDIDF	58	TYROSINASE 192-200
AFLPWHRLF	59	TYROSINASE 206-214
YMNGTMSQV	60	TYROSINASE 369-376
KTWGQYWQV	61	PMEL 17 (GP100) 154-162
ITDQVPFSV	62	PMEL 17 (GP100) 209-217
YLEPGPTVA	63	PMEL 17 (GP100) 280-288
LLDGTATLRL	64	PMEL 17 (GP100) 476-485
ELNEALELEK	65	p53 343-351
STPPPGTRV	66	p53 149-157
LLPENNVLSPL	67	p53 25-35
LLGRNSFEV	68	p53 264-272
RMPEAAPPV	69	p53 65-73
KIFGSLAFL	70	HER-2/neu 369-377
IISAVVGIL	71	HER-2/neu 654-662
CLTSTVQLV	72	HER-2/neu 789-797
YLEDVRLV	73	HER-2/neu 835-842
VLVKSPNHV	74	HER-2/neu 851-859
RFRELVSEFSRM	75	HER-2/neu 968-979
LLRLSEPAEL	76	PSA 119-128
DLPTQEPAL	77	PSA 136-144
KLQCVD	78	PSA 166-171
VLVASRGRAV	79	PSA 36-45
VLVHPQWVL	80	PSA 49-57
DMSLLKNRFL	81	PSA 98-107
QWNSTAFHQ	82	HBV envelope 121-130
VLQAGFF	83	HBV envelope 177-184
LLLCLIFL	84	HBV envelope 250-257
LLDYQGML	85	HBV envelope 260-267
LLVPFV	86	HBV envelope 338-343
SILSPFMPLL	87	HBV envelope 370-379
PLLPIFFCL	88	HBV envelope 377-385
ILSTLPETTV	89	HBV core 529-538
FLPSDFFPSV	90	HBV core 47-56

KLHLYSHPI	91	HBV polymerase 489-498
ALMPLYACI	92	HBV polymerase 642-651
HLYSHPIIL	93	HBV polym. 1076-1084
FLLSLGIHL	94	HBV polym. 1147-1153
HLLVGSSGL	95	HBV polymerase 43-51
GLSRYVARL	96	HBV polymerase 455-463
LLAQFTSAI	97	HBV polymerase 527-535
YMDDVVLGA	98	HBV polymerase 551-559
GLYSSTVPV	99	HBV polymerase 61-69
NLSWL	100	HBV polymerase 996-1000
KLPQLCTEL	101	HPV 16 E6 18-26
LQTTIHDII	102	HPV 16 E6 26-34
FAFRDLCIV	103	HPV 16 E6 52-60
YMLDLQPET	104	HPV 16 E7 11-19
TLHEYMLDL	105	HPV 16 E7 7-15
LLMGTLGIV	106	HPV 16 E7 82-90
TLGIVCPI	107	HPV 16 E7 86-93
LLMGTLGIVCPI	108	HPV 16 E7 82-93
LLMGTLGIVCPICSQK	109	HPV 16 E7 82-97

The nucleic acid in the microparticles described herein can be either distributed throughout the microparticle, or can be in a small number of defined regions within the microparticle. Alternatively, the nucleic acid can be in the core of a hollow core microparticle. The microparticle preferably does not contain a cell (e.g., a bacterial cell), or a naturally occurring genome of a cell, such as a naturally occurring intact genome of a cell.

The microparticles can also include a stabilizer compound (e.g., a carbohydrate, a cationic compound, a pluronic, e.g., Pluronic-F68 (Sigma-Aldrich Co., St. Louis, MO) or a DNA-condensing agent). A "stabilizer compound" is a compound that acts to protect the nucleic acid (e.g., to keep it supercoiled or protect it from degradation) at any time during the production of microparticles. Examples of stabilizer compounds include dextrose, sucrose, dextran, trehalose polyvinyl alcohol, cyclodextrin, dextran sulfate, cationic peptides, pluronics, e.g., Pluronic F-68 (Sigma-Aldrich Co., St. Louis, MO) and lipids such as hexadecyltrimethylammonium bromide. The stabilizer compound can remain associated with the DNA after a later release from the polymeric matrix.

The invention also features a preparation of microparticles comprising microparticles, such as the microparticles described herein. In some embodiments, at least 90% of the microparticles in the preparation have a diameter less than about 100 microns. In some cases, it is desirable for at least 90% of the microparticles to have a diameter less than about 60 microns, and preferably less than about 11 microns.

In another embodiment, the invention features a microparticle less than about 100 microns in diameter (e.g., about 100 microns, between 60 and 100 microns, less than about 60 microns, less than about 50 microns, less than about 40 microns, less than about 30 microns, less than about 20 microns, less than about 11 microns, less than about 5 microns, or less than about 1 micron), including a polymeric matrix and a nucleic acid molecule, wherein the nucleic acid molecule includes an expression control sequence operatively linked to a coding sequence. The expression product encoded by the coding sequence is a protein that, when expressed in a macrophage *in vivo*, downregulates an immune response, either specifically or in general. Examples of such proteins include tolerizing proteins, MHC blocking peptides, altered peptide ligands, receptors, transcription factors, and cytokines.

In some embodiments of the microparticles described herein, the nucleic acid need not encode a peptide, but could modulate an immune response by stimulating the release of γ-interferon, IL-12, or other cytokines, or by polyclonally activating B cells, macrophages, dendritic cells, or T cells. For example, poly I:C or CpG-containing nucleic acid sequences can be used (Klinman et al., Proc. Nat. Acad. Sci. (USA) 93:2879, 1996; Sato et al., Science 273:352, 1995).

In another embodiment, the invention features a process for preparing microparticles. A first solution, including a polymer dissolved in an organic solvent, is mixed (e.g., sonication, homogenization, vortexing, or microfluidization) with a second solution, which includes a nucleic acid dissolved or suspended in a polar or hydrophilic solvent (e.g., an aqueous buffer solution containing, for instance,

ethylenediaminetetraacetic acid, or tris(hydroxymethyl)aminomethane, or combinations thereof). Alternatively, a first solution, including a polymer dissolved in an organic solvent, is mixed (e.g., sonication, homogenization, vortexing, or microfluidization) with a powder that includes a nucleic acid, e.g., a lyophilized powder, a calcium precipitate, or a stabilizer-nucleic acid powder. The mixture forms a first emulsion. The first emulsion is then mixed with a third solution that can include a surfactant such as Pluronic, e.g., Pluronic F-68 (Sigma-Aldrich Co.), to form a second emulsion containing microparticles of polymer matrix and nucleic acid. The mixing steps can be executed, for example, in a homogenizer, vortex mixer, microfluidizer, or sonicator. Both mixing steps are carried out in a manner that minimizes shearing of the nucleic acid while producing microparticles on average smaller than 100 microns in diameter.

The second solution can, for example, be prepared by column chromatography and further purification of the nucleic acid (e.g., by ethanol or isopropanol precipitation), then dissolving or suspending the purified or precipitated nucleic acid in an aqueous, polar, or hydrophilic solution.

The first or second solution can optionally include a surfactant, a buffer, a DNA-condensing agent, or a stabilizer compound (e.g., 1-10% dextrose, trehalose, sucrose, dextran, or other carbohydrates, polyvinyl alcohol, cyclodextrin, hexadecyltrimethylammonium bromide, Pluronic F-68 (Sigma-Aldrich Co.,, St. Louis, MO), another lipid, or dextran sulfate) that can stabilize the nucleic acid or emulsion by keeping the nucleic acid supercoiled during encapsulation and throughout the microparticle formation.

The second emulsion is optionally mixed with a fourth solution including an organic solvent. The second emulsion can optionally be stirred (i.e., alone or as a mixture with the fourth solution) at an elevated temperature (e.g., room temperature to about 60°C), for example, to facilitate more rapid evaporation of the solvents.

Alternative ways to remove solvent include addition of alcohol, application of a vacuum, or dilution.

The procedure can include the additional step of washing the microparticles with an aqueous solution to remove organic solvent, thereby producing washed microparticles. The procedure can additionally include a step of concentrating the microparticle, e.g., by centrifugation, diafiltration, or sieving, e.g., in a SWECO unit. The washed microparticles can then be subjected to a temperature below 0°C, to produce frozen microparticles, which are in turn lyophilized to produce lyophilized microparticles. The microparticles can optionally be suspended in water or in an excipient, such as Tween-80, mannitol, sorbitol, or carboxymethyl-cellulose, prior to or after lyophilization (if any).

When desired, the procedure can include the additional step of screening the microparticles to remove those larger than 100, 60, 50, or 20 microns in diameter.

Still another embodiment of the invention features a preparation of microparticles that include a polymeric matrix, a proteinaceous antigenic determinant, and a DNA molecule that encodes an antigenic polypeptide that can be different from, or the same as, the aforementioned proteinaceous antigen determinant. The antigenic determinant contains an epitope that can elicit an antibody response. The antigenic polypeptide expressed from the DNA can induce a T cell response (e.g., a CTL response). The DNA can be plasmid DNA, and can be combined in the same microparticle as the antigenic determinant, or the two can be in distinct microparticles that are then mixed together. In some cases, an oligonucleotide, rather than a proteinaceous antigenic determinant, can be encapsulated together with a nucleic acid plasmid. Alternatively, the oligonucleotide may be encapsulated in a separate particle. The oligonucleotide can have antisense or ribozyme activity, for example.

In another embodiment, the invention features a method of administering nucleic acid to an animal by introducing into the animal (e.g., a mammal such as a human, non-human primate, horse, cow, pig, sheep, goat, dog, cat, mouse, rat, guinea, hamster, or

ferret) any of the microparticles described in the paragraphs above. The microparticles can be provided suspended in a aqueous solution or any other suitable formulation, and can be, for example, delivered orally, vaginally, rectally, or by inhalation, or injected or implanted (e.g., surgically) into the animal. They can optionally be delivered in conjunction with a protein such as a cytokine, an interferon, an antigen, or an adjuvant.

In another embodiment, the invention features a preparation of microparticles, each of which includes a polymeric matrix, a stabilizing compound, and a nucleic acid expression vector. The microparticles of the invention can each include a plurality of stabilizer compounds. The polymeric matrix includes one or more synthetic polymers having solubility in water of less than about 1 mg/l; in the present context, synthetic is defined as non-naturally occurring. At least 90% of the microparticles have a diameter less than about 100 microns. The nucleic acid can be either RNA or DNA. When present as RNA, in some embodiments at least 50% (and preferably at least 70% or even 80%) is in the form of closed circles. The nucleic acid can be a linear or circular molecule, and can thus be, e.g., a plasmid, or may include a viral genome, or part of a viral genome. The microparticles do not comprise a virus. When circular and double-stranded, it can be nicked, i.e., in an open circle, or super-coiled. In some embodiments the nucleic acids are plasmid molecules, at least 25% (and preferably at least 35%, 40%, 50%, 60%, 70%, or even 80%) of which are supercoiled.

The nucleic acid can also be an oligonucleotide, e.g., an antisense oligonucleotide or ribozyme.

The preparation can also include a stabilizer compound, e.g., dextrose, sucrose, dextran, trehalose polyvinyl alcohol, cyclodextrin, dextran sulfate, and cationic peptides.

In a further embodiment, the invention features a preparation of microparticles, each of which comprises a polymeric matrix, a nucleic acid molecule, and a lipid. The microparticles are not encapsulated in liposomes, and the microparticles do not comprise cells. By "do not comprise cells" is meant that the microparticles do not contain cells

(e.g., bacterial cells) and that the microparticle is not a cell. Preferably, the microparticle does not comprise a virus. It is understood that the microparticles may themselves be taken up by cells such as macrophages, as is explained above.

The nucleic acid in this embodiment may be any of the above-mentioned nucleic acid molecules and may also include an isolated nucleic molecule. By isolated nucleic acid molecule is meant any synthetic (including recombinant) nucleic acid molecule or a naturally occurring nucleic acid molecule removed from the virus or cell in which it is normally present.

The lipid can be, e.g., a cationic lipid, an anionic lipid, or a zwitterionic lipid, or may have no charge. Examples of lipids include cetyltrimethylammonium and phospholipids, e.g., phosphatidylcholine. The microparticles may contain one or more than one type of lipid, e.g., those lipids present in lecithin lipid preparations, and may also include one or more stabilizer compounds as described above.

In another embodiment, the invention includes a microparticle less than about 100 microns in diameter (e.g., about 100 microns, between 60 and 100 microns, less than about 60 microns, less than about 50 microns, less than about 40 microns, less than about 30 microns, less than about 20 microns, less than about 11 microns, less than about 5 microns, or less than about 1 micron), which includes a polymeric matrix, a lipid, and a nucleic acid molecule. The microparticle is not encapsulated in a liposome, and the microparticle does not comprise a cell.

The nucleic acid molecule in the microparticle can be circular, and the nucleic acid molecule may include an expression control sequence operatively linked to a coding sequence. The microparticle may optionally include a stabilizer compound or targeting molecule as described above.

In another embodiment, the invention includes a microparticle less than about 100 microns in diameter (e.g., about 100 microns, between 60 and 100 microns, less than about 60 microns, less than about 50 microns, less than about 40 microns, less than about

30 microns, less than about 20 microns, less than about 11 microns, less than about 5 microns, or less than about 1 micron), that preferably is not encapsulated in a liposome. The microparticle includes a polymeric matrix, a lipid, and a nucleic acid molecule that includes an expression control sequence operatively linked to a coding sequence. The coding sequence encodes an expression product that can include: (1) a polypeptide at least 7 amino acids in length, having a sequence essentially identical to the sequence of (a) a fragment of a naturally-occurring mammalian protein, or (b) a fragment of a naturally-occurring protein from an infectious agent that infects a mammal; (2) a peptide having a length and sequence that permit it to bind to an MHC class I or II molecule; and the polypeptide or peptide linked to a trafficking sequence. The expression product can additionally include an amino terminal methionine residue, and can also be immunogenic.

The expression product may include overlapping antigenic peptides derived from (1)(a) or (1)(b) or (2) above, e.g., two, three, four or more antigenic peptides arranged in series, where the sequence at the carboxy terminal end of the first forms a portion of the amino terminal end of the second, and a portion of the carboxy terminal end of the second forming a portion of the amino terminal end of the third, etc. An example of an amino acid sequence containing overlapping peptides is the amino acid sequence LLMGTLGIVCPIC (SEQ ID NO:110), which includes the MHC class I-binding peptides LLMGTLGIV (SEQ ID NO:111) and TLGIVCPIC (SEQ ID NO:115). For additional examples of amino acid sequences containing overlapping peptides, see, e.g., U.S. Patent 6,013,258 (herein incorporated by reference).

The expression product may alternatively or in addition include a polypeptide having two or more antigenic peptides, wherein the antigenic regions do not overlap. These tandem arrays of peptides may include two, three, four or more peptides (e.g., up to ten or twenty or more) that can be the same or different. Such tandemly arranged peptides can, of course, be interspersed with overlapping peptides. For examples of

polypeptides containing tandem arrays of peptides, e.g., antigenic peptides derived from human papilloma virus proteins, see U.S. Serial Number 60/154,665, filed September 16, 1999, and U.S. Serial Number 60/169,846, filed December 9, 1999 (herein incorporated by reference).

In some embodiments, the expression product (1) has an amino acid sequence that differs by no more than 25% from the sequence of a naturally occurring peptide recognized by a T cell; (2) is recognized by the T cell; and preferably (3) alters the cytokine profile of the T cell (e.g., an "altered peptide ligand").

The above expression product may include an MHC class II-binding amino acid sequence at least 50% identical to the sequence of a fragment of a protein at least 10 amino acids in length. The protein can be, e.g., myelin basic protein (MBP), proteolipid protein (PLP), invariant chain, GAD65, islet cell antigen, desmoglein, α-crystallin, or β-crystallin, or may be an amino acid sequence essentially identical to one or more of the sequences of SEQ ID NOS 1-46.

The above expression product can also include a trafficking sequence, e.g., a sequence that trafficks to endoplasmic reticulum, a sequence that trafficks to a lysosome, a sequence that trafficks to an endosome, a sequence that trafficks to an intracellular vesicle, or a sequence that trafficks to the nucleus. Such trafficking sequences include signal peptides (the amino terminal sequences that direct proteins into the ER during translation), ER retention peptides such as KDEL, and lysosome-targeting peptides such as KFERQ and QREFK, and other pentapeptides having Q flanked on one side by four residues selected from K, R, D, E, F, I, V, and L. Nuclear localization sequences include nucleoplasmin- and SV40-like nuclear targeting signals as described in Chelsky et al., *Mol. Cell Biol.*, 9:2487, 1989; Robbins, *Cell,* 64:615, 1991, and Dingwall et al., *TIBS,* 16:478, 1991. Some nuclear localization sequences include AVKRPAATKKAGQAKKK (SEQ ID NO:112), RPAATKKAGQAKKKKLD (SEQ ID NO:113), and AVKRPAATKKAGQAKKKLD (SEQ ID NO:114).

In other embodiments, the expression product can include an amino acid sequence essentially identical to the sequence of an antigenic portion of a tumor antigen, e.g., a tumor antigen from one of the proteins listed in Table 3.

The expression product may also include an amino acid sequence essentially identical to the sequence of an antigenic fragment of a protein naturally expressed by an infectious agent. The infectious agent can be, e.g., virus, a bacterium, or a parasitic eukaryote, e.g., a yeast. The infectious agent can thus include, e.g., human papilloma virus, human immunodeficiency virus, herpes simplex virus, hepatitis B virus, hepatitis C virus, *Plasmodium* species, mycobacteria, *Chlamydia*, and *Helicobacter* species.

In another embodiment, the expression product can include the amino acid sequence of a therapeutic protein. A "therapeutic protein" is an amino acid sequence, e.g., a full-length protein or a peptide derivative of the full-length protein, that is essentially identical to the amino acid sequence of a naturally occurring protein or a portion thereof. Preferably, the naturally occurring protein is naturally expressed in a human. When expressed in a subject, a therapeutic protein can affect a subject by a mechanism other than by presentation of the protein or a peptide thereof by an MHC molecule to a T cell. For example, the therapeutic protein can be an anti-inflammatory protein such as α-MSH. Alternatively, the therapeutic protein can be a cytokine such as IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, TGF-β, or γ-IFN. Alternatively, the therapeutic protein can be a growth factor such as erythropoietin, GM-CSF, G-CSF, PDGF, TPO, SCF, aFGF, bFGF, or insulin. The therapeutic protein can thus be any protein whose expression would be beneficial to a subject in need of treatment. In some embodiments, the expression product differs by no more than 25% from the sequence of a naturally occurring protein or a portion thereof.

Also included in the invention is a method of administering a nucleic acid to an animal (e.g., a human) by introducing the lipid-containing microparticles described above into the animal. The lipid particles may in addition include stabilizing agents. The

microparticles may be introduced via oral, mucosal, inhalation, or parenteral routes, e.g., by subcutaneous, intramuscular, or intraperitoneal injection.

In another embodiment, the invention includes a process for preparing lipid-containing microparticles. The steps include providing a first solution that contains a polymer dissolved in an organic solvent, and providing a second solution that includes a nucleic acid dissolved or suspended in a polar or hydrophilic solvent. The first and second solutions are mixed to form a first emulsion. The first emulsion is then mixed with a third solution to form a second emulsion. At least one of the first, second, and third solutions also includes a lipid or lipids. Both mixing steps are carried out in a manner that minimizes shearing of the nucleic acid while producing microparticles having an average diameter smaller than 100 microns.

The lipid or lipids can be included in either the first, second, or third solution, or in a combination of these solutions. In some embodiments the lipid is present in a concentration of 0.001 to 10.0%, or 0.1 to 1.0% (weight/volume), in one or more of the solutions.

The process may optionally include subjecting the microparticles to a temperature below 0°C, to produce frozen microparticles, and lyophilizing the frozen microparticles, to produce lyophilized microparticles.

The invention also includes a preparation of microparticles, each of which includes a polymeric matrix, a lipid, a proteinaceous antigenic determinant, an isolated nucleic acid molecule that encodes an antigenic polypeptide, and, optionally, a stabilizer agent.

Also included in the invention is a method of administering nucleic acid to an animal by providing a preparation of lipid-containing microparticles and introducing the preparation into the animal. The lipid-containing microparticles may optionally contain at least one stabilizer agent, e.g., a carbohydrate.

Also included in the invention is a method of administering a composition of the invention to an animal (e.g., a human) by a depot system. In a preferred embodiment, a composition of the invention is deposited at a target site, e.g., a site in a subject where drug delivery is desired, to produce a therapeutic effect at the target site. In another embodiment, a composition of the invention is deposited at a site distant from the target site, e.g., a site distant from the site in the subject where drug delivery is desired, to produce a therapeutic effect at the target site by systemic administration of a bioactive compound. The depot system can be adapted to release bioactive compounds over time. An example of a useful depot site is muscle tissue.

In another aspect, a composition is administered by a carrier system. A "carrier system" is a formulation that contains inclusion compounds, e.g., rotaxanes, cyclodextrins, or macrocycles, which can "contain" the bioactive compound. The inclusion compound functions as a "container" for a therapeutic compound.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present application, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

Brief Description of the Drawings

FIGS. 1A to 1C are a set of three plasmid maps, of the pvA2.1/4, luciferase, and VSV-Npep plasmids, respectively.

FIG. 2 is a plot of size distribution of DNA-containing microparticles as analyzed on a COULTER™ counter.

FIGS. 3A and 3B are a set of photographs of two agarose electrophoresis gels indicating degree of DNA supercoiling as a function of different homogenization speeds and durations.

FIG. 4 is a graph showing the release over time of DNA from microparticles prepared from DNA resuspended in TE or CTAB.

FIG. 5 is a graph showing the release over time of DNA from microparticles containing no lipid ("TE"), lecithin, or OVOTHIN™ 160.

FIG. 6 is a graph showing T cell responses from mice injected with lipid-containing microparticles containing luciferase-encoding DNA.

FIG. 7 is a graph depicting the time-course DNA release kinetics of microparticles containing either no lipid (A) or taurocholic acid (B).

FIG. 8 is a graph showing total serum anti-β gal IgG in Balb/c mice at 3 weeks, 6 weeks, and 12 weeks after a one shot immunization with 30 μg of β gal DNA encapsulated in PLGA microparticles (with or without lipid). Each bar represents mean values ± SE, as determined by β gal specific ELISA, of individual mice in groups of between 6- 9, and 2-3 for normal mouse serum (NMS).

FIG. 9 depicts serum anti-β gal IgG titers in Balb/c mice immunized once with 30 μg β gal DNA encapsulated in PLGA microparticles (with or without lipid). Antibody titers, as determined by β gal specific ELISA, are geometric mean titers ± SE of individual mice in groups of between 12-19.

FIG. 10 is a graph showing serum anti-β gal specific IgG isotypes in Balb/c mice immunized once with 30 μg DNA encapsulated in PLGA microparticles (with or without lipid).

FIG. 11 depicts MHC Class II restricted T cell proliferative responses to β Gal antigen in Balb/c mice 6 weeks after a one shot immunization with 30 μg DNA encapsulated in PLGA microparticles (with or without lipid) or blank PLGA microparticles (contained neither lipid nor DNA). Data are expressed as mean stimulation index ± SE of individual mice in groups of 9 tested in triplicate.

FIGS. 12A and 12B are graphs illustrating β-gal peptide-specific γ-IFN secretion response by Balb/c T cells from immunized mice.

FIGS. 13A and 13B are depictions of lungs that were harvested from a mouse vaccinated with pCMV/β-gal msp containing PEG-DSPE and challenged six weeks post-immunization with CT26.CL25 (FIG. 13A) and a non-vaccinated mouse that was similarly challenged (FIG. 13B). Tumor nodules are visible against normal (black) tissue.

FIG. 14 is a representation of three electrophoresis gels, showing pDNA integrity (% supercoiling) in hydrated PLG microparticles, without lipid (left panel), with PEG-DSPE (center panel), and with n-lauroyl sarcosine (right panel). In each panel, lane 1 corresponds to a 1 kb Marker; lane 2 corresponds to 250 ng input DNA; and lanes 3-8 correspond to the DNA after 1.5 hours, 1 day, 3 days, 8 days, 15 days, and 21 days, respectively.

FIG. 15 is a representation of an electrophoresis gel, showing the effects of DNase I on naked DNA, PLG-encapsulated pDNA microparticles without lipid, and PLG-encapsulated pDNA microparticles with PEG-DSPE at 30 minutes, 60 minutes, and 2 hours post-incubation, as indicated.

FIG. 16 is a copy of a micrograph of murine muscle tissue, showing microparticle-mediated β-galactosidase expression, day 10, using PEG-DSPE-containing microparticles.

FIGS. 17A and 17B, respectively, are graphs showing serum levels of SEAP (ng/ml) over time and percentage of animals in different groups at various time points expressing > 0.3 ng/ml of serum secreted alkaline phosphatase (SEAP).

FIG. 18 is a graph showing the kinetics of serum SEAP expression (ng/ml) as a function of different dose regimen. P values are from two-sided student t test.

FIG. 19 is a graph of serum SEAP levels (ng/ml) as a function of time for single (diamond) and multiple (square) microparticle injections.

FIG. 20 is a graph of optical density versus dilution, indicating binding of antibodies after immunization of mice with large microparticles (black bars), small microparticles (white bars), and normal sera (grey bars).

Detailed Description of the Invention

Compositions of the invention contain a delivery matrix, an anionic or zwitterionic compound, and a bioactive agent, e.g. a peptide, protein, and/or nucleic acid.

Examples of anionic compounds useful in the invention include polyethylene glycol diacyl phosphatidyl ethanolamine, taurocholic acid, taurodeoxycholic acid, chrondoitin sulfate, alkyl phosphocholines, alkyl-glycero-phosphocholines, phosphatidylserine, phosphotidylcholine, phosphotidylinositol, cardiolipin, lysophosphatide, sphingomyelin, phosphatidylglycerols, phosphatidic acid, diphytanoyl derivatives, glycocholic acid, cholic acid, and N-lauroyl sarcosine.

Anionic lipids can be used as the anionic compound of the composition. The anionic compound can be, e.g., a lipid sulfonate, lipid sulfate, lipid phosphonate, or lipid phosphate. Examples of lipids of the invention include polyethylene glycol diacyl ethanolamine, taurocholic acid, glycocholic acid, cholic acid, N-lauroyl sarcosine, and phosphatidylinositol.

Examples of zwitterionic compounds of the invention include CHAPSO (3-3-(Cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate), CHAPS ((3-3-(Cholamidopropyl)dimethylammonio]- 1-propanesulfonate, poly(AMPS) (poly(2-acrylamido-2-methyl-1-propanesulfonic acid), and phosphatidylethanolamine.

The composition can be constructed such that the anionic or zwitterionic compound is a component of the delivery matrix. Examples of delivery matrices of the invention that contain an anionic or zwitterionic compound as a component include a synthetically modified phosphonate derivatized macrocycle, a synthetically modified sulfonate derivatized macrocycle, a synthetically modified phosphonate derivatized cyclodextrin, and a synthetically modified sulfonate derivatized cyclodextrin.

The compositions of the invention are formulated in one of two ways: (1) to maximize delivery into the patient's phagocytic cells, or (2) to form a deposit in the tissues of the patient, from which the nucleic acid is released gradually over time; upon release, the nucleic acid is taken up by neighboring cells (including antigen presenting cells (APCs) and/or muscle cells.

The compositions of the invention can be used in the manufacture of a medicament for the treatment of, for example, cancer, any of the autoimmune diseases listed in Table 1, infectious disease, inflammatory disease, or any other condition treatable with a particular defined nucleic acid. Phagocytosis of compositions by macrophages, dendritic cells, and other APCs is an effective means for introducing the nucleic acid into these cells.

The compositions can be delivered directly into the bloodstream (i.e., by intravenous or intraarterial injection or infusion) where uptake by the phagocytic cells of the reticuloendothelial system (RES) is desired. Alternatively, the compositions can be delivered orally, into mucosally sites, nasally, vaginally, rectally or intralesionally. The compositions can also be delivered via subcutaneous injection, to facilitate take-up by the phagocytic cells of the draining lymph nodes. Alternatively, the compositions can be introduced intradermally (i.e., to the APCs of the skin, such as dendritic cells and Langerhans cells) or intramuscularly. Finally, the compositions can be introduced into the lung (e.g., by inhalation of powdered microparticles or of a nebulized or aerosolized solution or suspension containing the microparticles), where the compositions are picked up by the alveolar macrophages.

Once a phagocytic cell phagocytoses the compositions, the nucleic acid is released into the interior of the cell. Upon release, it can perform its intended function: for example, expression by normal cellular transcription/translation machinery (for an expression vector), or alteration of cellular processes (for antisense or ribozyme molecules).

Because these compositions are passively targeted to macrophages and other types of professional APC and phagocytic cells, they represent a means for modulating immune function. Macrophages and dendritic cells serve as professional APCs, expressing both MHC class I and class II molecules. In addition, the mitogenic effect of DNA can be used to stimulate non-specific immune responses mediated by B, T, NK, and other cells.

Delivery, via the compositions of the invention, of an expression vector encoding a foreign antigen that binds to an MHC class I or class II molecule will induce a host T cell response against the antigen, thereby conferring host immunity.

Where the expression vector encodes a blocking peptide (See, e.g., WO 94/04171) that binds to an MHC class II molecule involved in autoimmunity,

presentation of the autoimmune disease-associated self peptide by the class II molecule is prevented, and the symptoms of the autoimmune disease alleviated.

In another example, an MHC binding peptide that is identical or almost identical to an autoimmunity-inducing peptide can affect T cell function by tolerizing or anergizing the T cell. Alternatively, the peptide could be designed to modulate T cell function by altering cytokine secretion profiles following recognition of the MHC/peptide complex. Peptides recognized by T cells can induce secretion of cytokines that cause B cells to produce antibodies of a particular class, induce inflammation, and further promote host T cell responses.

Induction of immune responses, e.g., specific antibody responses to peptides or proteins, can require several factors. It is this multifactorial nature that provides impetus for attempts to manipulate immune related cells on multiple fronts, using the microparticles of the invention. For example, compositions can be prepared that carry both DNA and polypeptides within each compositions; alternatively, compositions can be prepared that carry either DNA or polypeptide, and then mixed. Dual-function microparticles are discussed below.

CTL Responses

Class I molecules present antigenic peptides to immature T cells. To fully activate T cells, factors other than the antigenic peptide are required. Full length proteins such as interleukin-2 (IL-2), IL-12, and gamma interferon (γ-IFN) promote CTL responses. These proteins or DNA encoding these proteins can be provided together with DNA encoding polypeptides that include CTL epitopes. The DNA encoding polypeptides that include CTL epitopes can encode a polypeptide having two or more antigenic peptides, wherein the antigenic regions do not overlap. These tandem arrays of peptides may include two, three, four or more peptides (e.g., up to ten or twenty or more) that can be the same or different. Such tandemly arranged peptides can be interspersed

with overlapping peptides. Alternatively, proteins that bear helper T (T_H) determinants can be included with DNA encoding the CTL epitope. T_H epitopes promote secretion of cytokines from T_H cells and play a role in the differentiation of nascent T cells into CTLs.

Alternatively, proteins, nucleic acids, or adjuvants that promote migration of lymphocytes and macrophages to a particular area could be included in microparticles along with appropriate DNA molecules. Uptake of the DNA is enhanced as a result, because release of the protein would cause an influx of phagocytic cells and T cells as the microparticle degrades. The macrophages would phagocytose the remaining microparticles and act as APC, and the T cells would become effector cells.

Antibody Responses

Elimination of certain infectious agents from the host may require both antibody and CTL responses. For example, when the influenza virus enters a host, antibodies can often prevent it from infecting host cells. However, if cells are infected, then a CTL response is required to eliminate the infected cells and to prevent the continued production of virus within the host.

In general, antibody responses are directed against conformational determinants and thus require the presence of a protein or a protein fragment containing such a determinant. In contrast, T cell epitopes are linear determinants, typically just 7-25 residues in length. Thus, when there is a need to induce both a CTL and an antibody response, the microparticles can include a DNA encoding an antigenic protein or both an antigenic protein and a DNA encoding a T cell epitope.

Slow release of the protein from microparticles would lead to B cell recognition and subsequent secretion of antibody, while phagocytosis of the microparticles would cause APCs (1) to express the DNA of interest, thereby generating a T cell response; and (2) to digest the protein released from the microparticles, thereby generating peptides that are subsequently presented by class I or II molecules. Presentation by class I or II

molecules promotes both antibody and CTL responses, since T_H cells activated by the class II/peptide complexes would secrete non-specific cytokines.

Immunosuppression

Certain immune responses lead to allergy and autoimmunity, and so can be deleterious to the host. In these instances, there is a need to inactivate tissue-damaging immune cells. Immunosuppression can be achieved with microparticles bearing DNA that encodes epitopes that down-regulate T_H cells or CTLs, e.g., blocking peptides and tolerizing peptides. Additionally, immunosuppression can be achieved with microparticles bearing DNA encoding TGF-β or αMSH. In these microparticles, the effect of the immunosuppressive DNA could be amplified by including certain proteins in the carrier microparticles with the DNA. A list of such proteins includes antibodies, receptors, transcription factors, and the interleukins.

For example, antibodies to stimulatory cytokines or homing proteins, such as integrins or intercellular adhesion molecules (ICAMs), can increase the efficacy of the immunosuppressive DNA epitope. These proteins serve to inhibit the responses of already-activated T cells, while the DNA further prevents activation of nascent T cells. Induction of T cell regulatory responses can be influenced by the cytokine milieu present when the T cell receptor (TCR) is engaged. Cytokines such as IL-4, IL-10, and IL-6 promote T_H2 differentiation in response to the DNA-encoded epitope. T_H2 responses can inhibit the activity of T_H1 cells and the corresponding deleterious responses that result in the pathologies of rheumatoid arthritis, multiple sclerosis and juvenile diabetes.

Inclusion of proteins comprising soluble forms of costimulatory molecules (e.g., CD-40, gp-39, B7-1, and B7-2), or molecules involved in apoptosis (e.g., Fas, FasL, Bc12, caspase, bax, TNFα, or TNFα receptor) is another way to inhibit activation of particular T cell and/or B cells responses. For example, B7-1 is involved in the activation of T_H1 cells, and B7-2 activates T_H2 cells. Depending on the response that is required,

one or the other of these proteins could be included in the microparticle with the DNA, or could be supplied in separate microparticles mixed with the DNA-containing microparticles.

Microparticles for Implantation

A second microparticle formulation of the invention is intended not to be taken up directly by cells, but rather to serve primarily as a slow-release reservoir of nucleic acid that is taken up by cells only upon release from the microparticle through biodegradation. The nucleic acid can be complexed to a stabilizer, e.g., to maintain the integrity of the nucleic acid during the slow-release process. The polymeric particles in this embodiment should therefore be large enough to preclude phagocytosis (i.e., larger than 5 μm and preferably larger than 20 μm). Such particles are produced by the methods described above for making the smaller particles, but with less vigorous mixing of the aforementioned first or second emulsions. That is to say, a lower homogenization speed, vortex mixing speed, or sonication setting can be used to obtain particles having a diameter around 100 μm rather than 5 μm. The time of mixing, the viscosity of the first emulsion, or the concentration of polymer in the first solution can also be altered to affect particle dimension.

The larger microparticles can be formulated as a suspension, a powder, or an implantable solid, to be delivered by intramuscular, subcutaneous, intradermal, intravenous, or intraperitoneal injection; via inhalation (intranasal or intrapulmonary); orally, e.g. in the form of a tablet; or by implantation. These particles are useful for delivery of any expression vector or other nucleic acid for which slow release over a relatively long term is desired: e.g., an antisense molecule, a gene replacement therapeutic, a means of delivering cytokine-based, antigen-based, or hormone-based therapeutic, or an immunosuppressive agent. The rate of degradation, and consequently of release, varies with the polymeric formulation. This parameter can be used to control

immune function. For example, one would want a relatively slow release for delivery of IL-4 or IL-10, and a relatively rapid release for delivery of IL-2 or γ-IFN.

Composition of Polymeric Particles

Polymeric material is obtained from commercial sources or can be prepared by known methods. For example, polymers of lactic and glycolic acid can be generated as described in US Patent No. 4,293,539 or purchased from Aldrich.

Alternatively, or in addition, the polymeric matrix can include polylactide, polyglycolide, poly(lactide-co-glycolide), polyanhydride, polyorthoester, polycaprolactone, polyphosphazene, proteinaceous polymer, polypeptide, polyester, or naturally occurring polymers such as alginate, chitosan, and gelatin.

Preferred controlled release substances that are useful in the formulations of the invention include the polyanhydrides, co-polymers of lactic acid and glycolic acid wherein the weight ratio of lactic acid to glycolic acid is no more than 4:1, and polyorthoesters containing a degradation-enhancing catalyst, such as an anhydride, e.g., 1% maleic anhydride. Since polylactic acid can take at least one year to degrade *in vivo*, this polymer should be utilized by itself only in circumstances where extended degradation is desirable.

Association of Nucleic Acid and Polymeric Particles

Polymeric particles containing nucleic acids can be made using a double emulsion technique. First, the polymer is dissolved in an organic solvent. A preferred polymer is polylactic-*co*-glycolic acid (PLGA), with a lactic/glycolic acid weight ratio of 65:35, 50:50, or 75:25. Next, a sample of nucleic acid suspended in aqueous solution is added to the polymer solution and the two solutions are mixed to form a first emulsion. The solutions can be mixed by vortexing, microfluidization, shaking, sonication, or homogenization. Most preferable is any method by which the nucleic acid receives the

least amount of damage in the form of nicking, shearing, or degradation, while still allowing the formation of an appropriate emulsion. For example, acceptable results can be obtained with a Vibra-cell model VC-250 sonicator with a 1/8" microtip probe, at setting #3, or by controlling the pressure in the microfluidizer, or by using an SL2T Silverson Homogenizer with a 5/8" tip at 10K.

During this process, water droplets (containing the nucleic acid) form within the organic solvent. If desired, one can isolate a small amount of the nucleic acid at this point in order to assess integrity, e.g., by gel electrophoresis.

Alcohol precipitation or further purification of the nucleic acid prior to suspension in the aqueous solution can improve encapsulation efficiency. Precipitation with ethanol resulted in up to a 147% increase in incorporated DNA and precipitation with isopropanol increased incorporation by up to 170%.

The nature of the aqueous solution can affect the yield of supercoiled DNA. For example, the presence of detergents such as polymyxin B, which are often used to remove endotoxins during the preparation and purification of DNA samples, can lead to a decrease in DNA encapsulation efficiency. It may be necessary to balance the negative effects on encapsulation efficiency with the positive effects on supercoiling, especially when detergents, surfactants, and/or stabilizers are used during encapsulation. Furthermore, addition of buffer solutions containing either tris(hydroxymethyl)aminomethane (TRIS), ethylenediaminetetraacetic acid (EDTA), or a combination of TRIS and EDTA (TE) resulted in stabilization of supercoiled plasmid DNA, according to analysis by gel electrophoresis. Ph effects are also observed. Other stabilizing compounds, such as dextran sulfate, dextrose, dextran, CTAB, polyvinyl alcohol, and sucrose, were also found to enhance the stability and degree of supercoiling of the DNA, either alone or in combination with the TE buffer. Combinations of stabilizers can be used to increase the amount of supercoiled DNA. Stabilizers such as charged lipids (e.g., CTAB), pluronics, e.g., Pluroinc F-68 (Sigma-Aldrich Co., St. Louis,

MO), cationic peptides, or dendrimers (*J. Controlled Release*, 39:357, 1996) can condense or precipitate the DNA. Moreover, stabilizers can have an effect on the physical nature of the particles formed during the encapsulation procedure. For example, the presence of sugars or surfactants during the encapsulation procedure can generate porous particles with porous interior or exterior structures, allowing for a more rapid exit of a drug from the particle. The stabilizers can act at any time during the preparation of the microparticles: during encapsulation or lyophilization, or both, for example.

The first emulsion is then added to an organic solution, allowing formation of microparticles. The solution can be comprised of, for example, methylene chloride, ethyl acetate, acetone, polyvinyl pyrrolidone (PVP) and preferably contains polyvinyl alcohol (PVA). Most preferably, the solution has a 1:100 to 8:100 ratio of the weight of PVA to the volume of the solution. The first emulsion is generally added to the organic solution with stirring in a homogenizer (e.g., a Silverson Model L4RT homogenizer (5/8" probe) set at 7000 RPM for about 12 seconds) or a microfluidizer.

This process forms a second emulsion that can be subsequently added to another organic solution with stirring (e.g., in a homogenizer, microfluidizer, or on a stir plate). Subsequent stirring causes the first organic solvent (e.g., dichloromethane) to be released and the microparticles to become hardened. Heat, vacuum, or dilution can in addition be used to accelerate evaporation of the solvent. Slow release of the organic solvent (e.g., at room temperature) can result in "spongy" particles, while fast release (e.g., at elevated temperature) results in hollow-core microparticles. The latter solution can be, for example, 0.05% w/v PVA. If sugar or other compounds are added to the DNA, an equal concentration of the compound can be added to the third or fourth solution to equalize osmolarity, effectively decreasing the loss of nucleic acid from the microparticle during the hardening process. The resultant microparticles are washed several times with water to remove the organic compounds. Particles can be passed through sizing screens to selectively remove those larger than the desired size. If the size of the microparticles is

not crucial, one can dispense with the sizing step. After washing, the particles can either be used immediately, frozen for later use, or be lyophilized for storage.

Larger particles, such as those used for implantation, can be obtained by using less vigorous emulsification conditions when making the first emulsion, as has already been described above at length. For example, larger particles can also be obtained by altering the concentration of the polymer, altering the viscosity of the emulsion, altering the particle size of the first emulsion (e.g., larger particles can be made by decreasing the pressure used while creating the first emulsion in a microfluidizer), or homogenizing with, for example, the Silverson homogenizer set at 5000 RPM for about 12 seconds.

The washed, or washed and lyophilized, microparticles can be suspended in an excipient without negatively affecting the amount of supercoiled plasmid DNA within the microparticles. Excipients such as carbohydrates, polymers, or lipids are often used in drug formulation, and here provide for efficient microparticle resuspension, act to prevent settling, and/or retain the microparticles in suspension. According to analysis by gel electrophoresis, excipients (including Tween 80, mannitol, sorbitol, and carboxymethylcellulose) have no effect on DNA stability or supercoiling, when included prior to or after lyophilization.

After recovery of the microparticles or suspension of the microparticles in an excipient, the samples can be frozen and lyophilized for future use.

Characterization of Microparticles

The size distribution of the microparticles prepared by the above method can be determined with a COULTER™ counter. This instrument provides a size distribution profile and statistical analysis of the particles. Alternatively, the average size of the particles can be determined by visualization under a microscope fitted with a sizing slide or eyepiece.

If desired, the nucleic acid can be extracted from the microparticles for analysis by the following procedure. Microparticles are dissolved in an organic solvent such as chloroform or methylene chloride in the presence of an aqueous solution. The polymer stays in the organic phase, while the DNA goes to the aqueous phase. The interface between the phases can be made more distinct by centrifugation. Isolation of the aqueous phase allows recovery of the nucleic acid. The nucleic acid is retrieved from the aqueous phase by precipitation with salt and ethanol in accordance with standard methods. To test for degradation, the extracted nucleic acid can be analyzed by HPLC or gel electrophoresis.

Intracellular Delivery of Microparticles

Microparticles containing DNA are resuspended in saline, buffered salt solution, tissue culture medium, or other physiologically acceptable carrier. For *in vitro*/*ex vivo* use, the suspension of microparticles can be added either to cultured adherent mammalian cells or to a cell suspension. Following a 1-24 hour period of incubation, those particles not taken up are removed by aspiration or centrifugation over fetal calf serum. The cells can be either analyzed immediately or recultured for future analysis.

Uptake of microparticles containing nucleic acid into the cells can be detected by PCR, or by assaying for expression of the nucleic acid. For example, one could measure transcription of the nucleic acid with a Northern blot, reverse transcriptase PCR, or RNA mapping. Protein expression can be measured with an appropriate antibody-based assay, or with a functional assay tailored to the function of the polypeptide encoded by the nucleic acid. For example, cells expressing a nucleic acid encoding luciferase can be assayed as follows: after lysis in the appropriate buffer (e.g., cell lysis culture reagent, Promega Corp, Madison WI), the lysate is added to a luciferin containing substrate (Promega Corp) and the light output is measured in a luminometer or scintillation counter. Light output is directly proportional to the expression of the luciferase gene.

If the nucleic acid encodes a peptide known to interact with a class I or class II MHC molecule, an antibody specific for that MHC molecule/peptide complex can be used to detect the complex on the cell surface of the cell, using a fluorescence activated cell sorter (FACS). Such antibodies can be made using standard techniques (Murphy et al. *Nature*, Vol. 338, 1989, pp. 765-767). Following incubation with microparticles containing a nucleic acid encoding the peptide, cells are incubated for 10-120 minutes with the specific antibody in tissue culture medium. Excess antibody is removed by washing the cells in the medium. A fluorescently tagged secondary antibody, which binds to the first antibody, is incubated with the cells. These secondary antibodies are often commercially available, or can be prepared using known methods. Excess secondary antibody must be washed off prior to FACS analysis.

One can also assay by looking at T or B effector cells. For example, T cell proliferation, cytotoxic activity, apoptosis, or cytokine secretion can be measured.

Alternatively, one can directly demonstrate intracellular delivery of the particles by using nucleic acids that are fluorescently labeled, and analyzing the cells by FACS or microscopy. Internalization of the fluorescently labeled nucleic acid causes the cell to fluoresce above background levels. Because it is rapid and quantitative, FACS is especially useful for optimization of the conditions for *in vitro* or *in vivo* delivery of nucleic acids. Following such optimization, use of the fluorescent label is discontinued.

If the nucleic acid itself directly affects cellular function, e.g., if it is a ribozyme or an antisense molecule, or is transcribed into one, an appropriate functional assay can be utilized. For example, if the ribozyme or antisense nucleic acid is designed to decrease expression of a particular cellular protein, the expression of that protein can be monitored.

In Vivo Delivery of Microparticles

Microparticles containing nucleic acid can be injected into mammals intramuscularly, intravenously, intraarterially, intradermally, intraperitoneally, or subcutaneously, or they can be introduced into the gastrointestinal tract or the respiratory tract, e.g., by inhalation of a solution or powder containing the microparticles, or swallowing a tablet or solution containing the microparticles. Alternatively, the microparticles can be introduced into a mucosal site such as the vagina, nose, or rectum. Expression of the nucleic acid is monitored by an appropriate method. For example, expression of a nucleic acid encoding an immunogenic protein of interest is assayed by looking for an antibody or T cell response to the protein.

Antibody responses can be measured by testing serum in an ELISA assay. In this assay, the protein of interest is coated onto a 96 well plate and serial dilutions of serum from the test subject are pipetted into each well. A secondary, enzyme-linked antibody, such as anti-human, horseradish peroxidase-linked antibody, is then added to the wells. If antibodies to the protein of interest are present in the test subject's serum, they will bind to the protein fixed on the plate, and will in turn be bound by the secondary antibody. A substrate for the enzyme is added to the mixture and a colorimetric change is quantitated in an ELISA plate reader. A positive serum response indicates that the immunogenic protein encoded by the microparticle's DNA was expressed in the test subject, and stimulated an antibody response. Alternatively, an ELISA spot assay can be employed.

T cell proliferation in response to a protein following intracellular delivery of microparticles containing nucleic acid encoding the protein is measured by assaying the T cells present in the spleen, lymph nodes, or peripheral blood lymphocytes of a test animal. The T cells obtained from such a source are incubated with syngeneic APCs in the presence of the protein or peptide of interest. Proliferation of T cells is monitored by uptake of ^{3}H-thymidine, according to standard methods. The amount of radioactivity

incorporated into the cells is directly related to the intensity of the proliferative response induced in the test subject by expression of the microparticle-delivered nucleic acid. A positive response indicates that the microparticle containing DNA encoding the protein or peptide was taken up and expressed by APCs *in vivo*.

The generation of cytotoxic T cells can be demonstrated in a standard ^{51}Cr release assay. In these assays, spleen cells or peripheral blood lymphocytes obtained from the test subject are cultured in the presence of syngeneic APCs and either the protein of interest or an epitope derived from this protein. After a period of 4-6 days, the effector cytotoxic T cells are mixed with ^{51}Cr-labeled target cells expressing an epitope derived from the protein of interest. If the test subject raised a cytotoxic T cell response to the protein or peptide encoded by the nucleic acid contained within the microparticle, the cytotoxic T cells will lyse the targets. Lysed targets will release the radioactive ^{51}Cr into the medium. Aliquots of the medium are assayed for radioactivity in a scintillation counter. Assays, such as ELISA or FACS, can also be used to measure cytokine profiles of responding T cells.

Lipid-Containing Microparticles

As described above for anionic and zwitterionic lipid-containing compositions, the microparticles described herein can also include one or more types of lipids. The inclusion of a lipid in a microparticle can increase the stability of the nucleic acid in the microparticle, e.g., by maintaining a covalently closed double-stranded DNA molecule in a supercoiled state. In addition, the presence of a lipid in the particle is believed to modulate, i.e., increase or decrease, the rate at which a drug or nucleic acid is released from the microparticle.

Addition of a lipid to the microparticle can in certain cases increase the efficiency of encapsulation of the nucleic acid or increase the loading of the nucleic acid within microparticles. For example, the encapsulation efficiency may be improved because the

presence of the lipid reduces the surface tension between the inner aqueous phase and the organic phase. Reduction of the surface tension is thought to create an environment more favorable for the nucleic acid, and therefore to increase its retention within the microparticle. A reduction in surface tension also allows for the primary emulsion to be formed with less manipulation, which minimizes shearing of the nucleic acid and increases encapsulation efficiency. It is also possible that the presence of lipid in the microparticle may enhance the stability of the microparticle/nucleic acid formulation, and may increase the hydrophobic nature of the microparticles, thereby increasing uptake by phagocytic cells.

The lipids can be cationic, anionic, or zwitterionic, or may carry no charged groups, such as nonpolar glycerides. The lipids preferably are not present as liposomes that encapsulate (i.e., surround) the microparticles. The lipids may optionally form micelles.

Examples of lipids that can be used in the microparticles include acids (such as carboxylic acids), bases (such as amines), phosphatidylethanolamine, phosphatidyl glycerol, phosphatidyl serine, phosphatidyl inositol, phosphatidylcholine, phosphatidic acid, containing one or more of the following groups: propanoyl (trianoic), butyroyl (tetranoic), valeroyl (pentanoic), caproyl (hexanoic), heptanoyl (heptanoic), caproyl (decanoic), undecanoyl (undecanoic), lauroyl (dodecanoic) tridecanoyl (tridecanoic), myristoyl (tetradecanoic), pentadecanoyl (pentadecanoic), palmitoyl (hexadecanoic), phytanoyl (3,7,11,15-tetramethylhexadecanoic), heptadecanoyl (heptadecanoic), stearoyl (octadecanoic), bromostearoyl (dibromostearoic), nonadecanoyl (nonadecanoic), arachidoyl (eicosanoic), heneicosanoyl (heneicosanoic), behenoyl (docosanoic), tricosanoyl (tricosanoic), lignoceroyl (tetracosanoic), myristoleoyl (9-*cis*-tetradecanoic), myristelaidoyl (9-*trans*-tetradecanoic), palmitoleoyl (9-*cis*-hexadecanoic), palmitelaidoyl (9-*trans*-hexadecenoic), petroselinoyl (6-*cis*-octadecenoic), oleoyl (9-*cis*- octadecenoic), elaidoyl (9-*trans*-octadecenoic), linoleoyl (9-*cis*-12-*cis*-octadecadienoic), linolenoyl (9-

cis-12-*cis*-15-*cis* octadecadoenoic), eicosenoyl (11-*cis*-eicosenoic), arachidonoyl (5,8,11,14 (all *cis*) eicosatetraenoic), erucoyl (13-*cis*-docsenoic), and nervonoyl (15-*cis*-tetraosenoic).

Other suitable lipids include cetyltrimethyl ammonium, which is available as cetyltrimethyl ammonium bromide ("CTAB").

More than one lipid can be used to make a lipid-containing microparticle. Suitable commercially available lipid preparations include lecithin, OVOTHIN 160™, and EPIKURON 135F™ lipid suspensions, all of which are available from Lucas Meyer, Inc., Decatur, IL.

The lipid may also be isolated from an organism, e.g., a mycobacterium. The lipid is preferably a CD1-restricted lipid, such as the lipids described in Pamer, Trend Microbiol. 7:13, 1999; Braud, Curr Opin. Immunol. 11:100, 1999; Jackman, Crit. Rev. Immunol. 19:49, 1999; and Prigozy, Trends Microbiol. 6:454, 1998.

In addition to the lipids incorporated into the microparticles, the microparticles can be suspended in a lipid (or lipid suspension) to improve delivery, e.g., by injection.

The relative increase or decrease in release observed will depend in part on the type of lipid or lipids used in the microparticle. Examples of lipids that increase the release of nucleic acid from microparticles include CTAB and the lecithin and OVOTHIN™ lipid preparations.

The chemical nature of the lipid can affect its spatial relationship with the nucleic acid in the particle. If the lipid is cationic, it may interact directly with the nucleic acid. If the lipid is not charged, it may be interspersed within the microparticle.

The lipid-containing microparticles may also include the stabilizers described above. The inclusion of a lipid in a microparticle along with a stabilizer such as sucrose can provide a synergistic increase in the release of nucleic acids within the microparticle.

Lipid-containing microparticles can be prepared by adding a lipid to either the organic solvent containing the polymer, to the aqueous solution containing the DNA

solution, or to the third solution used to make the second emulsion, as described above. The solubility properties of a particular lipid in an organic or aqueous solvent will determine which solvent is used.

Some lipids or lipid suspensions can be added to either the organic solvent or aqueous solution. However, the release properties of the resulting microparticles can differ. For example, microparticles prepared by adding a lecithin lipid suspension to the aqueous nucleic acid-containing solution release amounts similar to or less than the amount released by microparticles prepared without lipids. In contrast, addition of the lecithin lipid suspension to the organic solvent produces microparticles that release more nucleic acid.

Microparticles may in addition be resuspended in a lipid-containing solution to facilitate resuspension and dispersion of the microparticles.

In addition to the lipid-containing microparticles described herein, microparticles may also be made using other macromolecules such as chitin, gelatin, or alginate, or various combinations of these macromolecules and lipids. These microparticles made with these other macromolecules may in addition include the above-described stabilizing agents.

The following are examples of the practice of the invention. They are not to be construed as limiting the scope of the invention in any way.

EXAMPLES

Example 1: Incorporation of DNA; Analysis of Particle Size and DNA Integrity

Preparation of DNA for Incorporation

Plasmid DNA was prepared by standard methods using MEGA-PREP® Kit (Qiagen) according to the manufacturer's instructions. An endotoxin-free buffer kit (Qiagen) was used for all DNA manipulations. The DNA was resuspended in distilled, deionized, sterile water to give a final concentration of 3 μg/μl. FIG. 1 shows plasmid maps of DNA expression vectors encoding a) luciferase, b) a vesicular stomatitis virus

(VSV) peptide epitope termed VSV-Npep, and c) a human papilloma virus (HPV) peptide epitope termed A2.1/4.

Association of DNA with PLGA

200 mg of poly-lactic-*co*-glycolic acid (PLGA) (Aldrich, 65:35 ratio of lactic acid to glycolic acid) was dissolved in 5-7 ml of methylene chloride. 300 µl of the DNA solution prepared above, containing 900 µg DNA, was added to the PLGA solution. The mixture was sonicated in a Model 550 SONIC DISMEMBRATOR™ (Fisher Scientific) on setting #3 for 5-60 seconds, and the resulting emulsion was analyzed. An emulsion verified to contain particles of desired size having DNA of satisfactory integrity (as determined below) was added to a beaker containing 50 ml aqueous 1% w/v polyvinyl alcohol (PVA) (mw range: 30-70 kdal). The mixture was homogenized in a POWERGEN® homogenizer (Fisher Scientific) set at 3000-9000 RPM for 5-60 seconds. Again, the DNA integrity was analyzed. In the cases where the DNA was found to be sufficiently intact, the resulting second emulsion was transferred into a second beaker containing 100 ml aqueous 0.05% PVA, with constant stirring. The stirring was continued for 2-3 hours.

The microparticle solution was poured into a 250 ml centrifuge tube and spun at 2000 rpm for 10 minutes. The contents of the tubes were decanted and the sedimented particles were resuspended in 100 ml deionized water. After repeating the centrifugation and decanting steps, the particles were frozen in liquid nitrogen and finally lyophilized until dry.

Analysis of Microparticle Size Profile

5 mg of the lyophilized microparticles were resuspended in 200 µl water. The resulting suspension was diluted to about 1:10,000 for analysis with a COULTER™ counter. FIG. 2 is a print-out from the COULTER™ counter that indicates that approximately 85% of the microparticles were between 1.1 and 10 µm in diameter.

Determination of DNA Integrity

2-5 μg of the microparticles were wet with 10 μl water in an EPPENDORF™ tube. 500 μl chloroform was added with thorough mixing to dissolve the polymeric matrix. 500 μl water was added, again with mixing. The resulting emulsion was centrifuged at 14,000 rpm for 5 minutes. The aqueous layer was transferred to a clean EPPENDORF™ tube, along with 2 volume equivalents of ethanol and 0.1 volume equivalents of 3M aqueous sodium acetate. The mixture was centrifuged at 14,000 rpm for 10 minutes. After aspiration of the supernatant, the pelleted DNA was resuspended in 50 μl water. DNA was electrophoresed on a 0.8% agarose gel next to a standard containing the input DNA. The DNA on the gel was visualized on a UV light box. Comparison with the standard gives an indication of the integrity of the microparticles' DNA. The microparticle formation procedure was deemed successful if the incorporated DNA retained a high percentage of supercoiled DNA relative to the input DNA.

As indicated in FIGS. 3A and 3B, homogenization speed and duration are inversely related to DNA integrity. FIG. 3A depicts the DNA isolated from microparticles prepared by homogenization at 7000 rpm for 1 minute (lane 1), and supercoiled input DNA (lane 2). FIG. 3B shows DNA isolated from microparticles prepared by homogenization at 7000 rpm for 5 seconds (lane 1), DNA isolated from microparticles prepared by homogenization at 5000 rpm for 1 minute (lane 2), and supercoiled input DNA (lane 3).

Example 2: Preparation of DNA and Microparticles

DNA preparation

500 ml bacterial cultures were poured into one liter centrifuge bottles. The cultures were centrifuged at 4000 rpm at 20°C for 20 minutes. The media were poured off from the pelleted bacteria. The bacterial pellet was completely resuspended in 50 ml buffer P1 (50mM Tris-Hcl, Ph 8.0; 10mM EDTA; 100 μg/ml RNAse), leaving no clumps. 50 ml of buffer P2 (200 Mm NaOH, 1% SDS) was added with gentle swirling, and the suspensions were incubated at room temperature for five minutes. 50 ml of buffer P3 (3.0 M potassium acetate, Ph 5.5, chilled to 4°C) was added with immediate, gentle mixing. The suspensions were incubated on ice for 30 minutes, and then centrifuged at 4000 rpm at 4°C for 30 minutes.

A folded, round filter was wetted with water. When the centrifugation was complete, the supernatant was immediately poured through the filter. The filtered supernatant was collected in a clean 250 ml centrifuge bottle.

15 ml of Qiagen ER buffer was added to the filtered lysate, mixing by inverting the bottle 10 times. The lysate was incubated on ice for 30 minutes.

A Qiagen-tip 2500 column was equilibrated by applying 35 ml QBT buffer (750 Mm sodium chloride; 50 Mm MOPS, Ph 7.0; 15% isopropanol; and 0.15% triton X-100). The column was allowed to empty by gravity flow. The incubated lysate was applied to the column and allowed to enter by gravity flow. The column was washed with 4 x 50 ml Qiagen Endofree QC buffer (1.0 M NaCl; 50 Mm MOPS, Ph 7.0; 15% isopropanol). The DNA was eluted from the column with 35 ml of QN buffer (1.6 M NaCl,; 50 Mm MOPS, Ph 7.0; 15% isopropanol) into a 50 ml polypropylene screwcap centrifuge tube. The DNA suspension was split into two tubes by pouring approximately 17.5 ml of the suspension into a second 50 ml screwcap tube.

Using a sterile 10 ml pipet, 12.25 ml isopropanol was added to each tube. The tubes were closed tightly and thoroughly mixed. The contents of each tube were poured

into 30 ml Corex (VWR) centrifuge tubes. Each Corex tube was covered with PARAFILM®. The tubes were centrifuged at 11,000 rpm at 4°C for 30 minutes.

The supernatant was aspirated from each tube and the pellet was washed with 2 ml 70% ethanol. The ethanol was aspirated off. The pellet was air dried for 10 minutes, then resuspended in 0.5-1.0 ml water, and transferred to a sterile 1.5 ml microfuge tube.

Preparation of Microparticles

200 mg PLGA was dissolved in 7 ml methylene chloride in a 14 ml culture tube. A Fisher Scientific PowerGen 700 homogenizer equipped with a 7 mm mixing head was set to setting 6 and the speed 4.5. A Fisher Scientific Sonic Dismembrator 550 sonicator was set to setting 3.

1.2 mg of DNA in 300 µl H_2O was added to the PLGA solution and the resulting mixture was sonicated for 15 seconds. 50 ml of 1.0% PVA was poured into a 100 ml beaker and placed under the homogenizer. The homogenizer probe was immersed until it was about 4 mm from the bottom of the beaker and the homogenizer was supplied with power. The DNA/PLGA mixture was immediately poured into the beaker and the resultant emulsion was homogenized for 10 seconds. The homogenate was poured into the beaker containing 0.05% PVA.

The resulting emulsion was stirred for two hours, poured into a 250 ml conical centrifuge, and spun at 2000 rpm for 10 minutes. The pelleted microparticles were washed with 50 ml water, transferred to a 50 ml polypropylene centrifuge tube, and spun at 2000 rpm for 10 minutes. The pellet was washed with another 50 ml water and spun again at 2000 rpm for 10 minutes. The pellet was frozen in liquid nitrogen, then lyophilized overnight.

Extraction of DNA from microparticles for gel analysis

One milliliter of microparticles suspended in liquid were removed to a 1.5 ml microfuge tube and spun at 14,000 rpm for 5 minutes. Most of the supernatant was

removed. 50 µl of TE buffer (10 Mm Tris-Hcl, Ph 8.0; 1 Mm EDTA) was added and the microparticles were resuspended by flicking the side of the tube.

To isolate DNA from freeze-dried or vacuum-dried microparticles, 2-4 mg microparticles were weighed out into a 1.5 ml microfuge tube. 70 µl TE buffer was added, and the microparticles were resuspended.

200 µl chloroform was added to each tube and the tubes were vigorously, but not violently, shaken for two minutes to mix the aqueous and organic layers. The tubes were centrifuged at 14,000 rpm for 5 minutes. 30 µl of the aqueous phase was carefully removed to a new tube.

PicoGreen and Gel Analysis of Microparticles

3.5-4.5 mg microparticles were weighed out into a 1.5 ml microfuge tube. 100 µl DMSO was added to each tube, and the tubes were rotated at room temperature for 10 min. The samples were removed from the rotator and visually inspected to verify that the samples were completely dissolved. Where necessary, a pipet tip was used to break up any remaining clumps. None of the samples were allowed to remain in DMSO for more than 30 minutes.

For each sample to be tested, 990 µl TE was pipetted into three separate microfuge tubes. 10 µl of the DMSO/microparticle solution was pipetted into each 990 µl TE with mixing. The mixtures were centrifuged at 14,000 rpm for 5 minutes.

For each sample, 1.2 ml TE was aliquoted into a 5 ml round bottom snap cap centrifuge tube. 50 µl of the 1 ml TE/DMSO/microparticle mixture to the 1.2 ml TE. 1.25 ml of PicoGreen (Molecular Probes, Eugene, OR) reagent was added to each tube, and the fluorescence was measured in a fluorimeter.

Example 3: Alcohol Precipitation

Ethanol precipitation

DNA was prepared as in Example 2. Three samples, each containing 1.2 mg DNA, were precipitated by the addition of 0.1 vol 3 M sodium acetate and 2 volumes of ethanol. The DNA was resuspended in water to a final concentration of 4 mg/ml. DNA in two of the samples was resuspended immediately before use, and DNA in the third sample was resuspended and then rotated for 4 hours at ambient temperature. Control DNA at 4mg/ml was not ethanol precipitated.

Each of the four samples was encapsulated into microparticles by the procedure described in Example 2. The amount of DNA per mg of microparticles was determined by PicoGreen analysis, as described in Example 2. The following results were obtained:

Sample	mg of MS	µg DNA/mg MS	% incorp.	% incr.
Ethanol, 0 hr #1	**4.66**	**3.37**	**56**	**44**
Ethanol, 0 hr #2	**4.45**	**4.91**	**82**	**62**
Ethanol, 4 hr	**3.96**	**4.30**	**72**	**57**
Unprecip.	**3.97**	**1.85**	**31**	-

The results indicate that ethanol precipitation of DNA prior to encapsulation in microparticles resulted in increased incorporation ranging from 31% to greater than 56%, representing a 44-62% increase in the amount of encapsulated DNA.

The following experiments verify that the ethanol-precipitation effects observed above are independent of DNA preparation procedures.

DNA was prepared at three different facilities. Sample #1 was prepared as in Example 2. Sample #2 was prepared as in Example 2, but without the addition of ER-

removal buffer. Sample #3 was prepared in a scaled-up fermentation manufacturing run. The three DNA samples were representative of two different plasmids (DNA-1 and DNA-3 were identical) of sizes 4.5 kb and 10 kb. The three DNA samples were tested for the enhancement of encapsulation efficiency by ethanol precipitation. Three samples of DNA, each containing 1.2 mg, were precipitated by the addition of 0.1 vol 3 M sodium acetate and 2 volumes ethanol. The DNA was resuspended in water at a concentration of 4 mg/ml. Three control DNA samples, at 4mg/ml, were not ethanol precipitated.

Each of the samples was encapsulated by the procedure described in Example 2.

The amount of DNA per mg of microparticles was determined by PicoGreen analysis as described in Example 2. The following results were obtained:

Sample	mg of MS	μg DNA/mg MS	% incorp.	% incr.
#1 eth. ppt.	3.35	3.10	67	59
#2 eth. ppt.	4.45	4.91	66	47
#3 eth. ppt.	3.34	2.65	48	29
#1 unppt.	3.38	1.95	42	-
#2 unppt.	3.35	1.80	45	-
#3 unppt.	3.33	1.81	37	-

The data show that ethanol precipitation increased the amount of DNA encapsulated in microparticles by 29-59%. The effect was demonstrated to hold regardless of size and preparation technique.

Isopropanol vs. ethanol precipitation

Plasmid DNA was precipitated with ethanol or isopropanol, then resuspended in water for 4 hours or 16 hours. Control DNA was not precipitated. Microparticles were made according to the protocol in Example 2. The following results were obtained:

Sample	mg of MS	μg DNA/mg MS	% incorp.	% incr.
unppt. #1	4.43	0.99	17	-
unppt. #2	4.30	0.99	17	-
eth. ppt. #1 16 hr	4.26	2.12	37	118
eth. ppt. #2 16 hr	4.34	1.66	31	82
isopro. ppt. #1 16 hr	4.60	1.71	31	82
isopro. ppt. #2 16 hr	4.90	1.72	32	88
eth. ppt. #1 4 hr	4.65	2.22	42	147
eth. ppt. #2 4 hr	4.27	1.69	30	76
isopro. ppt. #1 4 hr	4.55	1.41	25	47
isopro. ppt. #2 4 hr	4.30	2.78	46	170

These data demonstrate that alcohol precipitation increased the encapsulation efficiency of DNA, independent of the type of alcohol used to precipitate DNA and independent of the time following DNA precipitation.

Conductivity

The conductivities of the ethanol-precipitated and non-precipitated DNA samples were determined using a conductivity meter. It was found that precipitation of the DNA led to a decrease in the amount of salt present. The conductivity without ethanol precipitation was 384 μΩ, while the conductivity after ethanol precipitation was 182 μΩ.

Thus, alcohol precipitation, or any other means of salt/contaminant removal is likely to increase encapsulation efficiency. It therefore appears that treatments that render DNA free from contaminants are likely to increase the efficiency of DNA encapsulation.

DNA was then ethanol precipitated or precipitated in the presence of 0.4M NaCl and 5% hexadecyltrimethylammonium bromide (CTAB). The DNA was then encapsulated as described above. The DNA was extracted and analyzed by agarose gel electrophoresis. The results indicated that precipitation of the DNA with CTAB led to a marked increase in the amount of supercoiled DNA within the microparticles. However, this was accompanied by a decrease in the encapsulation efficiency (6%, rather than 26%).

Example 4: Addition of Stabilizer Compounds

TE buffer

Plasmid DNA was resuspended in TE buffer following ethanol-precipitation, in an attempt to increase DNA stability. The microparticles were then prepared as described in Example 2. DNA was extracted from the microparticles and analyzed by agarose gel electrophoresis. One lane was loaded with the input plasmid (pIiPLPLR); another lane with the plasmid DNA following ethanol precipitation, resuspension in water, and encapsulation in microparticles; and still another lane with the plasmid DNA following ethanol precipitation, resuspension in TE buffer, and encapsulation in microparticles. The results indicated that the amount of supercoiled DNA within microparticles was increased by resuspension in TE buffer.

Two other plasmids, designated pbkcmv-n-p and E3PLPLR, were subjected to the conditions described above. This experiment confirmed that the two other plasmids were also stabilized by the TE buffer.

The following experiment was conducted to determine the timing of the TE effect. 2 g PLGA was dissolved in 18 ml methylene chloride. 500 μg DNA was ethanol-

precipitated and dissolved in 3.6 ml TE or water. The two solutions were mixed by inverting several times and then sonicated in the Fisher apparatus (see Example 2) on setting 3 for 10 seconds with a 1/8" microtip. At various times after sonication (i.e., 5, 15, 30, 45, and 60 minutes), a 1 ml sample was removed from each tube, 100 µl water was added, the sample was centrifuged in an Eppendorf centrifuge, and the top layer of the centrifugated sample removed to a separate tube. The samples were then analyzed by gel electrophoresis.

The results indicated that TE buffer acted to stabilize the DNA early in the encapsulation process, during formation of the oil in water emulsion.

To determine the effect of Tris and/or EDTA in the TE buffer, DNA was resuspended in water, TE buffer, 10 Mm TRIS, or 1 Mm EDTA prior to encapsulation in microparticles by the method of Example 2. The DNA was extracted from the microparticles and analyzed on an agarose gel. Tris and EDTA were each found to be similar to the complete TE buffer in their ability to protect DNA during the encapsulation process and during lyophilization.

An experiment was carried out to determine the effect of pH on encapsulation (the pH of the EDTA, Tris, and TE solutions in the previous experiment were all similar). Microparticles were made by encapsulating DNA that had been ethanol precipitated and resuspended in Tris of different pH, or in phosphate buffered saline (PBS). The DNA was extracted after lyophilization of the particles, and analyzed on agarose gel. The results indicated that there was a significant pH effect on the stability of encapsulated DNA. Resuspension of the DNA in water (pH 6.5), PBS (pH 7.3), and Tris (pH 6.8) all led to a decrease in the ratio of supercoiled DNA relative to total DNA within the microparticles. Increasing the pH to 7.5 or higher had a positive effect on the amount of supercoiling, suggesting that basic pH levels are important for maintaining DNA stability. Increased pH also had an effect on encapsulation efficiency:

SAMPLE	mg of MS	µg DNA/mg MS	% incorp.
Tris pH 6.8	2.42	2.77	55.5
Tris pH 7.5	2.52	2.73	54.6
Tris pH 8.0	2.49	3.29	65.9
Tris pH 9.9	2.46	3.81	76.3
water	2.46	2.48	49.7
PBS pH 7.3	2.49	0.55	11
TE pH 8.0	2.52	2.22	44.3

Other Buffer Compounds

Borate and phosphate buffers were also tested for their effect on the quality of encapsulated DNA. DNA was ethanol precipitated, resuspended in various buffer solutions, and encapsulated according to the procedure of Example 2. The DNA was extracted from the microparticles and analyzed by agarose gel electrophoresis. TE, BE, and PE all afforded greater than 50% supercoiling in the encapsulated DNA. An added benefit to DNA was also discovered, resulting from EDTA in the presence of Tris, borate, or phosphate.

Other Stabilizer Compounds

In addition to buffers, other compounds were tested for their ability to protect the DNA during the encapsulation procedure. Plasmid DNA was ethanol-precipitated and resuspended in water or a solution of dextran sulfate. Microparticles were then prepared according to the method of Example 2. DNA was extracted from the microparticles before and after lyophilization and analyzed by agarose gel electrophoresis.

The results suggested that the addition of a stabilizer led to encapsulation of more supercoiled DNA than did resuspension of DNA in water alone. The greatest improvement in DNA structure was observed with a 10% dextran sulfate solution.

Protection apparently occurred at two levels. An effect of dextran sulfate was seen on DNA pre-lyophilization, as, following encapsulation, a greater proportion of DNA remained in the supercoiled state with increasing amounts of dextran sulfate. The protection rendered by the stabilizer also occurred during the lyophilization procedure, since the presence of the stabilizer during this process increased the percentage of DNA remaining in the supercoiled state.

To determine whether or not the effects of TE and other stabilizers were additive, ethanol-precipitated DNA was resuspended in TE or water, with or without a solution of another stabilizer (e.g., sucrose, dextrose, or dextran). Microparticles were prepared according to the method of Example 2. DNA was extracted from the microparticles and analyzed by agarose gel electrophoresis.

The results demonstrated that resuspending DNA in a stabilizer/TE solution is slightly better or equivalent to the use of TE alone, insofar as a greater percentage of DNA remains in the supercoiled state after encapsulation under these conditions.

Stabilizers were also added in combination, to determine whether or not the stabilizer effects are additive. DNA was ethanol-precipitated and resuspended in various stabilizer solutions. The DNA was encapsulated as described in Example 2, extracted, and analyzed by agarose gel electrophoresis. The results indicate that combinations of stabilizers can be used to increase the amount of encapsulated, supercoiled DNA.

Example 5: Addition of Excipients

To determine whether or not excipient compounds have an adverse effect on encapsulated plasmid DNA, microparticles were prepared from ethanol-precipitated DNA following the protocol in Example 2, with the exception that prior to lyophilization, the microparticles were resuspended in solutions containing excipients. Each sample was then frozen and lyophilized as in Example 2. The final concentration of the excipients in the microparticles upon resuspension at 50 mg/ml was 0.1% Tween 80, 5% D-sorbitol,

5% D-mannitol, or 0.5% carboxymethylcellulose (CMC). DNA was extracted from the microparticles and analyzed on an agarose gel.

The results illustrated that addition of excipients prior to lyophilization did not significantly affect DNA stability or the degree of supercoiling.

EXAMPLE 6: Treatment with Microparticles Containing DNA

According to the procedure of Example 1, microparticles are prepared containing DNA encoding a peptide having an amino acid sequence about 50% identical to PLP residues 170-191 (SEQ ID NO: 2). A multiple sclerosis patient whose T cells secrete excess T_H1 cytokines (i.e., IL-2 and γ-IFN) in response to autoantigens is injected intravenously with 100 µl to 10 ml of the microparticles. Expression of the PLP-like peptide by APCs results in the switching of the cytokine profile of the T cells, such that they instead produce T_H2 cytokines (i.e., IL-4 and IL-10) in response to autoantigens.

Example 7: Tolerizing with Microparticles Containing DNA

According to the procedure of Example 1, microparticles are prepared containing DNA encoding a peptide having an amino acid sequence corresponding to MBP residues 33-52 (SEQ ID NO: 34). A mammal is injected subcutaneously with 1-500 µl of the microparticles. Expression of the MBP peptide by APCs results in the tolerization of T cells that recognize the autoantigen.

Example 8: Implantation of Microparticles

A DNA molecule, including an expression control sequence operatively linked to a sequence encoding both a trafficking sequence and a peptide essentially identical to myelin basic protein (MBP) residues 80-102 (SEQ ID NO: 1), is associated with a polymer to form microparticles, according to the procedure of Example 1. Particles smaller than 100 µm are removed. The polymeric constituent of the microparticle is

poly-lactic-*co*-glycolic acid, where the ratio of lactic acid to glycolic acid is 65:35 by weight. The resulting microparticles are surgically implanted subcutaneously in a patient.

Example 9: Preparation of Microparticles Containing Both DNA and Protein

Plasmid DNA is prepared by standard methods using MEGA-PREP® Kit (Qiagen) according to the manufacturer's instructions. An endotoxin-free buffer kit (Qiagen) is used for all DNA manipulations. The DNA is resuspended in distilled, deionized, sterile water to give a final concentration of 3 μg/μl. Additionally, 0-40 mg of purified protein is added to about 1 ml of the DNA solution. A mass of gelatin, equal to about 20% of the mass of protein, is added.

Up to 400 mg of PLGA (i.e., at least ten times the mass of protein) is dissolved in about 7 ml methylene chloride. The DNA/protein solution is poured into the PLGA solution and homogenized or sonicated to form a first emulsion. The first emulsion is poured into about 50-100 ml of an aqueous solution of surfactant (e.g., 0.05% to 2% PVA by weight). The mixture is homogenized at about 3000-8000 RPM to form a second emulsion. The microparticles are then isolated according to the procedure of Example 1.

Example 10: Treatment with Microparticles Containing Both DNA and Protein

Microparticles including both an antigenic protein having the conformational determinants necessary for induction of B cell response against hepatitis B virus (HBV) and DNA encoding the CTL epitope for HBV are prepared according to the procedure of Example 8. A patient infected or at risk of infection with HBV is immunized with the microparticles.

Slow release of the protein from non-phagocytosed microparticles leads to B cell recognition of the conformational determinants and subsequent secretion of antibody. Slow release of the DNA or phagocytosis of other microparticles causes APCs (1) to

express the DNA of interest, thereby generating a T cell response; and (2) to digest the protein released from the microparticles, thereby generating peptides that are subsequently presented by class I or II molecules. Presentation by class I molecules promotes CTL response; presentation by class II molecules promotes both antibody and T cell responses, since T_H cells activated by the class II/peptide complexes secrete non-specific cytokines.

The results are elimination of HBV from the patient and continued prevention of production of virus within the patient's cells.

Example 11: Phagocytosis of Microparticles Containing Plasmid DNA by Murine Dendritic Cells

Microparticles were prepared by the procedure of Example 2, except that a fluorescent oligonucleotide was added during the encapsulation procedure. Splenic dendritic cells were isolated from mice and incubated with nothing, with fluorescent beads, or with the prepared microparticles. FACS analysis of the cells indicated that the fluorescent beads and the prepared microparticles were both phagocytosed. Moreover, the prepared microparticles did not fluoresce unless they had been ingested by the dendritic cells, suggesting that following phagocytosis, the microparticles became hydrated and degraded, allowing release the encapsulated DNA into the cell cytoplasm.

Example 12: Preparation of Lipid-Containing Microparticles

To prepare lipid-containing microparticles, 200 mg PLGA was dissolved in 7 ml of methylene chloride ("DCM") (J.T. Baker, Catalog # 9324-11) in a 14 ml tube. The resulting PLGA/DCM solution was poured into a 35 ml polypropylene cylindrical tube prepared by truncating a 50 ml polypropylene cylindrical tube at the 35 ml mark. An OVOTHIN™ lipid solution was added to the PLGA/DCM solution to a final concentration of 0.05% (vol/vol).

A Silverson SL2T homogenizer (East Longmeadow, MA) with a 5/8 inch slotted mixing head was preset at setting 10. Prior to beginning homogenization, 50 ml of a 1.0% PVA solution (Average MW: 23,000: 88% hydrolyzed) was poured into a 100 ml beaker, and 100 ml of 0.05% PVA/300 Mm sucrose solution was poured into a 250 ml beaker containing a 1.5-inch stir bar. The beaker was placed on a stir plate.

1.2 mg of pBVKCMluc DNA in 300 µl TE/10% SDS was added to the PLGA/DCM solution. The mixture was homogenized for 2 min. at room temperature to form a DNA/PLGA emulsion. The homogenizer was then shut off and the DNA/PLGA emulsion removed. The 1.0% PVA solution (50 Ml) was placed under the homogenizer probe, and homogenization resumed. The DNA/PLGA emulsion was immediately poured into the beaker containing the 1.0% PVA solution, and the mixture homogenized for 1 minute. The mixture was then poured into the beaker containing 0.05% PVA on the stir plate and stirred for two hours.

After two hours, the mixture was poured into a 250 Ml conical centrifuge tube and spun in a Beckman GS6R clinical centrifuge at 2500 rpm for 10 min. The pelleted microparticles were washed twice with water.

After the second washing the pellet was resuspended in water, frozen in liquid nitrogen and lyophilized for at least 11 hours.

DNA from microparticles prepared using TE/sucrose was present in a concentration of 2.33µg/ml (DNA/PLGA) and 55% supercoiling, whereas DNA from microparticles prepared using OVOTHIN™ lipid was present at a concentration of 1.66 µg/ml and 60% supercoiling.

Example 13: Preparation of Phosphatidylcholine-Containing Microparticles Containing CMVluc DNA

pBKCMVluc plasmid DNA was precipitated in ethanol and resuspended in a solution of TE Ph 8.0/10% sucrose. A lecithin lipid preparation (Lucas Meyer, Catalog

No. LECI-PC35F), which is enriched in phosphatidylcholine ("PC"), was added to the DNA solution in varying amounts (vol/vol) as indicated in Tables 5 and 6.

The lipid preparation initially formed a large aggregate after addition to the DNA solution. The aggregate was dispersed into smaller aggregates following vortexing for 20 seconds. After gentle agitation for 30 minutes at room temperature, the PC formed a colloidal suspension.

Lecithin-containing microparticles were formed by adding the suspension to a PLGA/DCM solution and proceeding as described in Example 12, above. The observed diameters for the microparticles ranged from 1-10μm.

Tables 5 and 6 provide the concentration of plasmid DNA in the microparticle (expressed in micograms of DNA per mg of polymeric material), the percent supercoiling (SC), and the percentage of starting plasmid DNA encapsulated in microparticles made using DNA resuspended in TE or TE plus 10% sucrose and various concentrations of lecithin. Final concentrations are shown.

TABLE 5

	μg/mg	%SC	% encap
10% sucrose TE Ph 8.0	2.79	55	46.5
0.3 μl (0.1% lecithin)	2.78	55	46.3
1.5 μl (0.5% lecithin)	2.55	55	42.5
3 μl (1.0% lecithin)	2.67	55	44.5

TABLE 6

	μg/mg	%SC
TE	2.39	40
1% lecithin/TE	2.7	40
5% lecithin/TE	1.56	50
10% lecithin/TE	1.23	50

Table 5 demonstrates that addition of lecithin to an initial concentration of 0-1.0% did not significantly affect properties of the encapsulated DNA, as indicated by the final

concentration of DNA in the particle, the percent supercoiling, or the percent of DNA encapsulated.

Table 6 reveals that lecithin present at an initial concentration of 5% or 10% resulted in increased supercoiling and a lower concentration of DNA relative to microparticles prepared using no lecithin or 1% lecithin.

Example 14: *In vitro* Release Properties of Lipid Microparticles

The amount of DNA released from microparticles was determined by preparing microparticles containing DNA and then resuspending the microparticles in an aqueous medium and assaying the supernatant for the presence of DNA using the indicator dye PicoGreen.

Approximately 150 mg of microparticles prepared in TE alone or in TE with CTAB were dissolved in 15 ml TE and injected into a Slide-A-Lyser ™ membrane (M.W. cut off, 10,000), which was then placed in 1 liter of TE at 37°C and stirred. Samples were removed with a syringe at time points, and a 75 µl aliquot of was centrifuged at 14k rpm for 5 min. Supernatant was removed and a fraction of this was assayed using PicoGreen.

FIG. 4 shows the percentage of DNA released over time from microparticles prepared using DNA resuspended in TE or CTAB. The percentage of DNA released from TE microparticles increased from slightly less than 20% after 7 days to about 40% after 42 days. In contrast, the percentage of DNA released from CTAB microparticles increased from about 60% after 7 days to over 80% after 42 days. These data demonstrate that CTAB increases the amount of DNA released from microparticles.

Release of DNA from lipid-containing microparticles was also examined in microparticles prepared using TE, TE/10% sucrose, 0.04% lecithin, and 0.04% OVOTHIN™ 160 lipid. Microparticles containing plasmid DNA were resuspended in TE, and release was assayed by PicoGreen analysis.

FIG. 5 shows the percentage of DNA released with time from microparticles prepared using the various lipids. The percentage of DNA released from microparticles prepared using 0.04% lecithin or 0.04% OVOTHIN™ 160 was about 80% after 50 days.

In contrast, about 20% of the DNA was released after 50 days from microparticles prepared using TE, and about 60% of DNA was released from microparticles prepared using 10% sucrose/TE. These results demonstrate that the presence of lipid in the microparticles increases the amount of DNA released from the microparticles.

Example 15: T Cell Proliferation Assays Following Administration of Lipid-Containing Microparticles

Balb/c mice were injected intravenously with 200 µl of microparticles containing the PBKCMVluc plasmid and OVOTHIN™ lipid preparation. Spleens were harvested 11 weeks after injection and analyzed by a T cell proliferation assay.

RBC were lysed and splenocytes washed, counted, and plated in RPMI media containing 10% FCS at $5x10^5$ or $2.5x10^5$ cells/well in 96 well flat bottom plates.

Luciferase antigen (Promega Corp, Madison WI) was added at concentrations ranging from 1 to 50 µg/ml. Studies were conducted using either 250,000 or 500,000 cells per well. The cells were incubated at 37°C for 5 days, after which H^3 thymidine was added to each well. 24 hours after addition of H^3 thymidine, the cells were harvested on a TOMTEC™ cell harvester and their radioactivity determined.

The results from the studies are shown in FIG. 6. Antigen-proliferative responses were detected using both 250,000 cells and 500,000 cells. These results demonstrate that the injected microparticles elicited a T cell response specific for the encoded luciferase.

Example 16: Production and Characterization of Microparticles Containing Anionic and Zwitterionic Lipids

Approximately 10.6 mg of plasmid DNA was dissolved in TE/sucrose buffer (with or without excipient), pH 8.0. The solution was emulsified by homogenization (Silverson L4R), then encapsulated by 1g of PLGA (Boehringer Ingelheim RG502, 12000 Da) /methylene chloride. The resulting emulsion was homogenized in a final aqueous phase (PVA, Air Products) and stirred at a controlled temperature. Microparticles thus generated were washed with deionized water, and lyophilized to obtain a white, flocculated powder. Sizing of the reconstituted microparticles was carried out on a Coulter Multisizer II to obtain size distributions.

Approximately 2.5 mg of lyophilized microparticles were reconstituted in 200 µl of TE buffer, pH 8.0. 500 µl of chloroform was added to dissolve the polymeric microparticles. The biphasic solution was rotated end-over-end at room temperature for 90 minutes to facilitate extraction of DNA into the aqueous phase. Concentrations of DNA (µg/mg) were measured at 260 nm by UV spectrophotometry.

Percent supercoiling of DNA in the microparticles after the encapsulation process was determined by gel agarose electrophoresis. Briefly, 250 ng of DNA was loaded onto the ethidium bromide/ agarose gel (bromothymol blue was used as the loading dye).

Residual poly(vinyl alcohol) (PVA) was determined by the following method: (a) exhaustive hydrolysis of 10 mg of microparticles by NaOH (5 ml), followed by neutralization by concentrated HCl (0.9 ml); (b) formation of the borate salt of poly(vinyl alcohol) by the addition of 3.7% boric acid (0.9 ml); (c) complexation of the borate salt by the addition of 0.1 ml of KI/I_2 solution (1.66% KI, 1,27% I_2); and (d) measurement of absorbance at 620 nm, and % PVA calculated using Beer-Lambert's Law.

Scanning Electron Micrographs (SEM) were obtained of the gold-sputtered microparticles (with and without excipient) using an AMR-1000 scanning electron microscope operated at an accelerating voltage of 10 kV.

Table 7 summarizes the physio-chemical properties of the lipid and non-lipid containing formulations. Addition of a lipid excipient to the formulation showed an improvement in DNA encapsulation values. Percent supercoiling was maintained at 85-95% post process, with and without addition of the excipient . SEMs of the microparticles showed uniform, spherical microparticles (1-10 μm) interdispersed with nanospheres (~400 nm).

TABLE 7: Physio-Chemical Characterization of the Microparticles

Lipid	**% super-coiled**	**DNA Encap-sulation**	**Size (n-avg)**	**Size (v-avg)**	**% PVA**	**% Lipid**
No lipid	90±5	3.68±0.6	2.29±1.0	5.50±1.9	0.91±0.14	none
PEG2K-DSPE	90 ±5	4.78±0.7	2.21±0.72	6.27±2.1	1.04±0.06	0.5±3.6
Taurocholic Acid	85±5	5.88±2.7	2.65±0.79	5.72±1.8	1.11±0.03	3.7±2.1
Glycocholic Acid	90±5	4.52±1.4	2.71±0.99	6.09±2.2	0.99±0.09	--
Cholic Acid	85±5	4.71±1.5	2.72±1.2	5.96±1.7	1.19±0.09	2.6±3.1
CHAPS	85±5	5.22±1.5	2.81±1.5	5.19±0.8	1.05±0.08	--
N-Lauroyl Sarcosine	95±10	4.21±2.1	2.63±0.7	5.66±1.1	0.89±0.03	--
Phosphatidyl Inositol	85±5	4.01±0.3	2.41±1.3	6.05±1.7	1.05±0.05	0.10

The amount of DNA released from microparticles was determined by preparing microparticles containing DNA and either anionic or zwitterionic lipids and then resuspending the microparticles in an aqueous medium and assaying the supernatant for the presence of DNA.

Approximately 2.5 mg of microparticles were weighed into 2 ml round bottomed centrifuge tubes and reconstituted with 1 ml Dulbecco's Phosphate Buffered Saline / 0.5 mM EDTA, pH 7.0. The tubes were rotated end over end in a 37°C incubator. Approximately 800 µl of supernatant was removed (n=3) at each of the following timepoints: 1 hour, 1 day, 3 days, 7 days, 10 days, 14 days, and 21 days. The removed supernatants were replaced with 800 µl of fresh PBS. Supernatants collected at each timepoint were analyzed for DNA content by a UV spectrphotometer (260 nm). The percent supercoiling of the DNA released at each timepoint was determined by agarose gel electrophoresis. pH measurements were carried out at each timpeoint, to ensure adequate buffering capacity of the release medium.

FIG. 7 compares the time-course DNA release kinetics of microparticles containing either no lipid (A) or taurocholic acid (B). pH measured during the course of the release experiments was between 6.7-7.0 for both formulations, demonstrating adequate buffering capacity of the release media as the micropspheres degraded over time. No significant differences were observed in DNA release kinetics between the lipid and non-lipid containing microparticles. Table 8 shows a lack of significant differences in DNA release kinetics between microparticles containing various lipid formulations.

TABLE 8: Total DNA Released at Day 1 from Microparticles Containing DNA and Anionic or Zwitterionic Lipids

Lipid	"Total DNA" at 1 day
PEG2K-DSPE	37.5 ±5.3
Taurocholic Acid	45.1 ±10.2
Glycocholic Acid	48.1 ±8.1
Cholic Acid	42.6 ±7.8
CHAPS	37.2 ±5.9
N-Lauroyl Sarcosine	35.1 ±9.3
Phosphatidyl Inositol	42.9 ±4.2

Example 17: *In Vivo* Immune Response Following Administration of Anionic Lipid-Containing Microparticles

DNA

An expression plasmid encoding the β-gal antigen driven by a CMV promoter was used in the experiments. Plasmid DNA used for immunization (see Example 16 for the production of microparticles) was prepared according to the manufacturer's instructions using an Endotoxin free Mega prep kit (Qiagen Corp; Chatsworth, CA).

Peptides

The synthetic peptide, TPHPARIGL, representing the naturally processed H-2L^d restricted epitope spanning amino acids 876-884 of β-gal and IPQSLDSWWTSL, the H-2L^d high binding epitope corresponding to residues S28-39 of hepatitis B surface Ag (HbsAg), were synthesized by Multiple Peptide Systems (San Diego, CA) to a purity of >90% as assessed by reverse phase high-pressure liquid chromatography (RP-HPLC). The identity of each of the peptides was confirmed by mass spectral analysis.

Cell Lines and Mice

The H-2^d mastocytoma cell line P815 (TIB-64) was obtained from the American Type Culture Collection (ATCC, Manassas, VA). Balb/c mice, 6-10 wk of age, were purchased from The Jackson Laboratory (Bar Harbor, ME).

Immunizations

For the humoral immune responses and the T cell proliferative responses, mice in groups of 3-6 were immunized once by intramuscular or intravenous injection with DNA formulations at week 0. The microparticle formulations were suspended in saline, at a dose of 30 μg DNA in 200 μl saline per animal. Fifty microliters of the formulations was injected in the tibialis anterior and 50 μl was injected in the hamstring of the two hind legs of each animal. In order to test reproducibility of microparticle batches these experiments were carried out 3 times with separately produced batches of microparticle formulations. The immunization protocol for the MHC Class I restricted T cell response assays included an identical boost injection given at week 2. Mice were bled from the retroorbital sinus and the sera were separated for the immunoassays.

T Cell Proliferation Assays

Mouse splenic T cells were purified using T cell enrichment columns (R& D Systems, Minneapolis, MN). *In vitro* Ag-stimulated T cell proliferation assays were performed with purified splenic T cells isolated 4 weeks after primary immunization with microparticles. The cultures were set up in U-bottomed 96-well plates. T cells ($2.5x\ 10^5$) were incubated with 50 μg/ml of β-gal antigen (Calbiochem Novabiochem, Pasadena, CA) in 200 μl of Eagle's Hanks' amino acid medium (Irvine Scientific, Santa Anna, CA) supplemented with 0.5% syngeneic mouse serum, 2mM glutamine, 100 U/ml penicillin, 100U/ml streptomycin, and $5x\ 10^{-5}$ M 2-ME. Syngeneic x-irradiated (3000 rad) splenocytes (5×10^5) were used as antigen presenting cells (APC). The cultures were incubated at 37^0C in a humidified atmosphere of 5% CO_2 and pulsed with 1 μCi of

[^{3}]TdR (sp. Act., 6.7 Ci/mmol; ICN, Irvine, CA) during the final 16 to 18 h, and harvested for liquid scintillation counting.

ELISA Assay

For the analysis of serum antibodies from mice immunized with β-gal DNA 96 well plates were incubated at room temperature for 3 hours with β-gal protein (Calbiochem Novabiochem, Pasadena, CA) at 2 μg/ml in phosphate buffered saline (PBS). Plates were washed and blocked by standard procedures (see, e.g., Harlow and Lane, "Immunoassay" in *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1988). The solid phase was incubated overnight at 4^{0}C with normal mouse serum (NMS) or antiserum, or β-gal specific mAb (Calbiochem Novabiochem, Pasadena, CA) followed by an incubation with horseradish peroxidase (HRP)-conjugated antibodies specific for mouse IgG (H+L). For isotype analysis, HRP-labeled goat anti-mouse IgG1 and IgG2a (Southern Biotechnology, Birmingham, AL) were used. The binding of antibodies was measured as absorbance at 405 nm after reaction of the immune complexes with ABTS substrate (Zymed, San Francisco, CA). Titers were defined as the highest dilution to reach an OD of 0.2.

γ-IFN was measured using a sandwich ELISA and the paired detection and capture antibodies and recombinant insect cell derived γ-IFN purchased from Pharmingen (San Diego, CA).

In Vitro Restimulation of Primed β-gal Specific MHC Class I Restricted T Cells

Spleens were removed from immunized mice 10 days after boosting. T cell enrichment was carried out as described earlier and these cells were incubated at 2 x 10^6/ml in RPMI tissue culture medium supplemented with 10 mM Hepes buffer, antibiotics and 10% v/v FCS (JRH BioSciences, Lenexa, KS) with x-irradiated (20,000 rad) β-gal peptide pulsed LPS/dextran stimulated syngeneic blasts at 2 x 10^6/ml in 24 well plates. Recombinant human IL2 (rhIL2) was added to these expansion cultures at

d2 at 10 U/ml and on d 5-6 these cells were used as responders in a cytokine release assay for detection of γ-IFN levels.

γ-IFN Release Assay

Co-culture was performed using P815 cells as stimulators that were pre-pulsed with 50 μM β-gal peptide or with the irrelevant peptide, H-2 L^d restricted epitope from HbsAg (to control for non specific γ-IFN release) pulsed P815 cells and *in vitro* restimulated primed T cells as effectors. Stimulators and effectors were set up in triplicate at a ratio of 1:1 and concentration of 1 x 10^6/ml for 24 hrs. Supernatants from these co-cultures were tested in duplicate for specific secretion of γ-IFN by ELISA. Data are presented after nonspecific subtraction as picograms of γ-IFN released by 1 x 10^5 effectors/ 24 hours.

(A) Humoral Immune Response

Specific serum immunoglobulin responses in mice immunized with 30 ug encapsulated DNA were measured by ELISA at 3, 6 and 12 weeks post immunization. Inclusion of an anioinic or zwitterionic lipid in the formulation resulted an increased incidence of humoral responses (Tables 9 and 10). In the intramuscular injected group (Table 9), inclusion of taurocholic acid or PEG-DSPE in the formulation increased the number of responders from 56% to 100% and 93%, respectively. In the intravenous injected group (Table 10), addition of lipid in the formulation resulted in 93% (taurocholic acid) and 87% (PEG-DSPE) responders vs. 47% in the group that received the no lipid formulation. Furthermore, inclusion of a lipid in the formulation resulted in the antibody response occurring faster (FIG. 8) with higher titers (FIG. 9). Analysis of the isotype of the antibody responses showed that the antibody response was primarily of the IgG2a isotype (FIG. 10), suggesting that DNA immunization with these formulations is a potent method for the generation of specific helper responses with a Th1-like phenotype. No specific antibodies were detected in the sera of blank immunized mice.

TABLE 9 - INCIDENCE OF SEROPOSITIVE MICE 6 WEEKS POST INTRAMUSCULAR DELIVERY OF B GAL + LIPID MICROSPHERES

	1st ROUND			2nd ROUND			3rd ROUND			OVERALL INCIDENCE	
	B GAL 17	B GAL 18		B GAL 19	B GAL 20		B GAL 21	B GAL 22			%
CHAPS 11% 60 ug	1/3	1/3	**2/6**	1/3	3/3	**4/6**	2/3	2/3	**4/6**	**10/18**	**56**
CHAPS 11% 30 ug	2/3		**2/3**	2/3	nd	**2/3**	1/3	nd	**1/3**	**5/9**	**56**
CHAPS 33%	1/3	1/3	**2/6**	0/3	1/3	**1/6**	1/3	4/4	**5/7**	**8/16**	**50**
Cholic Acid	1/3	3/3	**4/6**	3/3	2/3	**5/6**	1/3	2/3	**3/6**	**12/18**	**67**
N-L-Sarcosine	0/3	2/3	**2/6**	2/3	1/3	**3/6**	3/3	1/3	**4/6**	**9/18**	**50**
Taurocholic Acid	nd	nd	**nd**	3/3	3/3	**6/6**	3/3	2/3	**6/6**	**12/12**	**100**
PEG DSPE	2/3	3/3	**5/6**	3/3	nd	**3/3**	3/3	3/3	**6/6**	**14/15**	**93**
Capric Acid	1/3	nd	**1/3**	nd	nd	**nd**	nd	nd		**1/3**	**33**
Glycocholic Acid	1/3	3/3	**4/6**	1/3	2/3	**3/6**	1/3	0/3	**1/6**	**8/18**	**44**
Blanks	nd	0/3	**0/3**	nd	0/3	**0/3**	nd	0/3	**0/3**	**0/9**	**0**
w/o Lipid	0/3	1/3	**1/6**	2/3	2/3	**4/6**	3/3	2/3	**5/6**	**10/18**	**56**
Naked	3/3	3/3	**6/6**	3/3	3/3	**6/6**	3/3	3/3	**6/6**	**18/18**	**100**

TABLE 10 - INCIDENCE OF SEROPOSITIVE MICE 6 WEEKS POST INTRAVENOUS DELIVERY OF B GAL + LIPID MICROSPHERES

	1st ROUND			2nd ROUND			3rd ROUND			OVERALL INCIDENCE	
	B GAL 17	B GAL 18		B GAL 19	B GAL 20		B GAL 21	B GAL 22			%
CHAPS 11% 60 ug	2/3	2/3	4/6	1/3	2/3	3/6	3/3	3/3	6/6	13/18	72
CHAPS 11% 30 ug	4/4	nd	4/4	2/3	nd	2/3	3/3		3/3	9/10	90
CHAPS 33%	3/3	1/3	4/6	3/3	3/3	6/6	2/3	3/3	5/6	15/18	83
Cholic Acid	0/3	0/3	0/6	1/3	2/3	3/6	2/3	2/3	4/6	7/18	39
N-L-Sarcosine	0/3	2/3	2/6	2/3	2/3	4/6	0/3	2/3	2/6	8/18	44
Taurocholic Acid	nd		nd	3/3	36683	09-Sep	2/3	3/3	5/6	14/15	93
PEG DSPE	2/3	2/3	4/6	3/3	nd	3/3	3/3	3/3	6/6	13/15	87
Capric Acid	1/3	nd	1/3	nd	nd	nd	nd	nd	nd	1/3	33
Glycocholic Acid	0/3	0/3	0/6	2/3	2/3	4/6	1/3	1/3	2/6	6/18	33
Blanks	nd	0/3	0/3	nd	0/3	0/3	nd	0/3	0/3	0/9	0
w/o Lipid	1/3	0/3	1/6	1/3	3/3	4/6	2/3	2/4	4/7	9/19	47
Naked 30 ug	3/3	2/3	5/6	3/3	3/3	6/6	2/3	3/3	5/6	16/18	89
Naked 60 ug							2/3				

(B) Cell Mediated Responses

In order to investigate Class II MHC restricted CD4+ immune responses induced by the β-gal DNA formulations, purified T cells from the spleens of vaccinated mice were restimulated with antigen *in vitro*. Substantial T cell proliferation was observed, especially in the intramuscular treated groups (FIG. 11) in response to β-gal in cells from mice injected with DNA formulations containing either taurocholic acid or PEG-DSPE compared to cells from mice injected with formulations that did not include a lipid. The MHC Class II restricted T cell proliferative responses to β Gal antigen in Balb/c mice were measured 6 weeks after a one shot immunization with either 30 μg DNA encapsulated in PLGA microparticles (with or without lipid) or blank PLGA microparticles containing neither lipid nor DNA. Data are expressed as mean stimulation index ± SE of individual mice in groups of 9 tested in triplicate.

Example 18: Immunizations For MHC Class I Restricted T Cell Response Assays

The microparticle formulations were suspended in saline, at a dose of 30 μg DNA in 200 μl saline per animal. Fifty microliters of the formulation were injected in the tibialis anterior and 50 μl were injected into the hamstring in the two hind legs of each animal. The immunization protocol for the MHC Class I restricted T cell response assays included an additional boost injection given at week 2.

In vitro restimulation of primed β-gal specific MHC Class I restricted T cells

Spleens were removed from immunized mice 10 days after boosting. T cell enrichment was carried out as described earlier and these cells were incubated at 2 x 10^6/ml in RPMI tissue culture medium supplemented with 10 mM Hepes buffer, antibiotics and 10% v/v FCS (JRH BioSciences, Lenexa, KS) with x-irradiated (20,000 rad) β-gal peptide pulsed LPS/dextran stimulated syngeneic blasts at 2 x 10^6/ml in 24 well plates. Recombinant human IL2 (rhIL2) was added to these expansion cultures at d2

at 10 U/ml and on d 5-6 these cells were used as responders in a cytokine release assay for detection of γ-IFN levels.

γ-IFN release assay

Co-culture was performed using P815 cells as stimulators that were pre-pulsed with 50 μM β-gal peptide or with the irrelevant peptide, H-2 L^d restricted epitope from HBsAg (to control for non specific γ-IFN release) and *in vitro* restimulated primed T cells as effectors. Stimulators and effectors were set up in triplicate at a ratio of 1:1 and a concentration of 1 x 10^6/ml for 24 hrs. Supernatants from these co-cultures were tested in duplicate for specific secretion of γ-IFN by ELISA. The ratio of picograms of γ-IFN released by 1 x 10^5 effectors/24 hrs was calculated after subtraction of the media control. Average pg/ml values are representative of individual animals in two experiments.

The MHC Class I restricted T cell response as measured by γ-IFN release detected by ELISA from primed β-gal specific T cells is shown in FIGS. 12A and 12B, which illustrate β-gal peptide-specific γ–IFN secretion response by Balb/c T cells from immunized mice. The data indicate that the Class I response is not impaired by inclusion of PEG-DSPE in the particle formulation. To determine if lipid inclusion would make a significant difference if the DNA dose were reduced, animals were injected with decreasing amounts of formulated DNA. In this case, the data suggest that below a certain threshold level of DNA, lipid-containing formulations demonstrated enhanced class I restricted T cell responses.

To obtain the data shown in FIGS. 12A and 12B, peptide pulsed P815 cells were incubated with T cells following *in vitro* restimulation with peptide. FIG. 12A is based on data obtained from the experiment in which mice were immunized with two doses of PLGA microparticles (2 weeks apart) and splenocyte T cells responses were measured 10d after boosting. Each bar represents mean values ± SE of individual mice in groups of 4. FIG. 12B is based on data obtained from an experiment in which mice were

immunized once with titrating doses of DNA and T cell responses were measured 20 weeks later. Each bar represents values obtained from pools of 4 mice.

Example 19: *In Vivo* Protection Studies

For *in vivo* protection studies, mice were immunized with either DNA formulations that included or excluded PEG-DSPE 6 weeks before an i.v. challenge with 5×10^5 tumor cells as previously described. Mice were sacrificed on day 15, lungs were harvested and counting of lung metastases was carried out in a blinded fashion as previously described. In this method, once the mice were sacrificed, India ink solution was injected into the trachea, and the lungs were removed and bleached by immersion in Fekete's solution, rendering the lungs suitable for nodule enumeration (white against black background).

Protective immune responses have not previously been demonstrated following parenteral delivery of encapsulated DNA. To so demonstrate, we used a well-known tumor line expressing b-gal as a tumor antigen. Balb/c mice injected intramuscularly with 30 μg encapsulated β-gal DNA were challenged with either CT26.WT or CT26.CL25 tumor cell lines. As controls, non-immunized groups were also challenged with either the CT26.CL25 or CT26.WT cell lines. Examination of lungs harvested on day 15 after tumor inoculation indicated the presence of multiple pulmonary metastases in all mice challenged with the CT26.WT cell line. Immunized mice challenged with the CT26 β-gal expressing tumor (CT26.CL25) were protected from metastases and had completely clear lungs. Representative photographs of metastatic and tumor free lungs are shown in FIG. 13 to demonstrate the contrast between protected mice and those that developed tumor nodules (>200 per lung). These results demonstrate that encapsulated DNA vaccines delivered via a parenteral route elicit protective immune responses. FIGS. 13A and 13B, respectively, show photographs of lungs that were harvested from a mouse vaccinated with pCMV/β-gal msp containing PEG-DSPE and challenged six weeks post-

immunization with CT26.CL25 (FIG. 13A) and a non-vaccinated mouse that was similarly challenged (FIG. 13B). Tumor nodules are visible against normal (black) tissue.

Example 20: Determination of pDNA supercoiling in hydrated microparticles

Microparticles were extracted with chloroform and buffer to determine the percent supercoiling of the plasmid in the hydrated pellets over time. In the procedure used, 2.5 mg of PLG microparticles were weighed and resuspended with 200 µl of TRIS-EDTA buffer, pH 8.0. 500 µl of chloroform was added to the suspension to solubilize the microparticles. The mixture was rotated end-over-end for 90 minutes at ambient temperature to facilitate extraction of DNA from the organic (PLG/chloroform) phase into the aqueous supernatant. The samples were centrifuged at 14 krpm for 5 minutes. 100 µl of the supernatant was drawn off with a micro-tipped pipette. The quantity (µg) of DNA encapsulated in 1 mg of PLG was determined by UV spectrophotometry. As shown in FIG. 14, there was a substantial amount of supercoiled DNA left in the lipid-containing microparticles at 21 days, whereas DNA encapsulated in non-lipid containing microparticles had lost nearly all supercoiling at the end of 8 days.

Example 21: Protection of Encapsulated Microparticles From Endonucleases

Three samples of microparticles were incubated with 5 µg of DNase I in 10 mM Tris-HCl buffer containing 10 mM $MgSO_4$ (pH 8.0) for 30 minutes, 1 hour, and 2 hours, respectively, at 37°C. Following digestion, samples were analyzed by 0.8% agarose gel electrophoresis for DNA fragments. As shown in FIG. 15, DNA encapsulated in PEG-DSPE containing microparticles was protected from the nuclease, compared to DNA in non-lipid containing microparticles.

Example 22: ß-galactosidase expressed in muscle post-IM injection

PLG microparticles containing 25 μg β-gal DNA in 50 μl of PBS were injected into the anterior tibialis muscle of female BALB/c mice. Injected muscles were collected on day 6 post administration, fixed with 3 ml of 0.25% glutaraldehyde (J.T. Baker, Phillipsburg, NJ) at room temperature for 45 min, and then stained with X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; Promega, Madison, WI) solution at 37° C for 16 hrs with shaking. The stained muscles were post-fixed in 10% neutral buffered formalin for 24 hrs, photographed, and then sectioned and stained with histotoxylin and eosin. FIG. 16 shows microparticle-mediated expression in mouse muscle, day 10, achieved using microparticles containing PEG-DSPE as lipid excipient.

Example 23: Serum levels of bioactive protein following single intramuscular injection of plasmid DNA in microparticles

PLG microparticles containing mPEG-DSPE and pgWiz-SEAP DNA (Gene Therapy Systems, San Diego, CA), encoding for human secreted alkaline phosphatase, were re-suspended in saline and injected into the tibialis and hamstring muscles of 5-6-week old C57/Bl6 mice. Injection volumes were 50 μl/muscle. Mice were given either 50 or 100 μg DNA dose. Serum was collected via retro-orbital bleeding at different days post-injection and assayed for secreted bioactive SEAP using the Tropix Phospha-Light kit. The results are shown in FIGS. 17A and 17B. FIG 17A shows serum levels of SEAP (ng/ml) as a function of time. FIG. 17B indicates the percentage of animals in different groups at various time points expressing more than 0.3 ng/ml of serum SEAP.

Example 24: Serum levels of bioactive protein following intramuscular injection of plasmid DNA in microparticles

PLG microparticles containing mPEG-DSPE and pgWiz-SEAP DNA were re-suspended in saline and injected either once or on days 0 and 1 (2x) into the tibialis and hamstring muscles of C57/Bl6 mice (50 or 100 μg DNA per animal). Serum was collected at different days post-injection and assayed for secreted bioactive SEAP using the Tropix Phospha-Light kit. The results are provided in FIG. 18, which shows the kinetics of serum SEAP expression (ng/ml) as a function of different dose regimens. P values are from two-sided student t test.

Example 25: Serum Levels of Bioactive Protein Following Multiple Intramuscular Injection of Plasmid DNA in Microparticles

PLG microparticles containing mPEG-DSPE and pgWiz-SEAP DNA were re-suspended in saline and injected into the tibialis and hamstring muscles of C57/Bl6 mice (50 ml/muscle, 50 mg DNA per animal). Serum was collected at different days post-injection and assayed for secreted bioactive SEAP using the Tropix Phospha-Light kit. The results are provided in FIG. 19, which shows that SEAP expression can be sustained for more than 2 months by multiple injections of microparticles containing pSEAP. Numbers adjacent to data points indicate percentage of animals expressing more than 300 pg/ml of serum SEAP. Arrows indicate injection schedule. P values are calculated by a two-sided student t test.

Example 26: Total Serum IgG Titers in Balb/c mice immunized with β-Gal DNA encapsulated in PLG microspheres of size < 100m, compared with those of size <10m.

Balb/c mice in groups of 3-6 were immunized by a single intramuscular injection with pDNA-encapsulated microparticle formulations at week 0. The microparticles were suspended in saline, at a dose of 30 mg DNA in 200 ml saline per animal. 50 ml of the microparticle formulation was injected in the tibialis anterior (TA) and 50 ml was

injected in the hamstring muscle in each of the hind legs of each mouse. The mice were bled from the retro-orbital sinus and the sera were separated for the immunoassays.

For the analysis of serum antibodies from mice immunized with b-gal DNA, 96□well plates were incubated at room temperature for 3 hours with b-gal protein (Calbiochem Novabiochem, Pasadena, CA) at 2 mg/ml in phosphate buffered saline (PBS). Plates were washed and blocked by standard procedures. The solid phase was incubated overnight at 4°C with normal mouse serum (NMS) or antiserum, or b-gal specific mAb (Calbiochem Novabiochem, Pasadena, CA), and then incubated with horseradish peroxidase(HRP)-conjugated antibodies specific for mouse IgG (H+L). For isotype analysis, HRP-labelled goat anti-mouse IgG1 and IgG2a (Southern Biotechnology, Birmingham, AL) were used. The binding of antibodies was measured as absorbance at 405 nm after reaction of the immune complexes with ABTS substrate (Zymed, San Francisco, CA).

DNA contents of the microparticles were 4.5 mg/mg (<10 m) and 5.8 mg/mg (<100 m) extracted by an aqueous/organic method and assayed by UV spectrometry at 260 nm. The percent DNA supercoiling, determined by agarose gel electrophoresis was 90-95% for both categories of microparticles. Microparticle sizes measured by coulter sizing were 2-2.5 m (Navg) and 40-50 m (Navg), respectively. Total IgG titer of microparticles < 10m specific to β-galactosidase measured at 3 weeks by ELISA was approximately 1.5 times higher than that of the microparticles < 10m.

As shown in FIG. 20, the binding of antibodies was measured as absorbance at 405 nm after reaction of the immune complexes with ABTS substrate (Zymed, San Francisco, CA). Large microparticles (< 100 m; Large Msp) and smaller microparticles (< 10m; Msp), both containing PEG-DSPE, were both demonstrated to elucidate immune responses to β-gal antigen.

<u>Other Embodiments</u>

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, that the foregoing description is intended to illustrate and not limit the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.